ESA-SRB-ANZBMS 2021 ABSTRACTS

Contents

ORAL ABSTRACTS

1

Fibrodysplasia Ossificans Progressiva: Insights Into Heterotopic Bone Formation From a Rare Congenital Bone Disorder

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Fibrodysplasia ossificans progressiva (FOP) occurs in ~1 per 2 million population. It is characterized by congenital malformations of the great toes and progressive heterotopic ossification (HO) leading to immobility (1). In 2006, the disorder was associated with mutation in activin receptor 1A (ACVR1, also known as ALK2), a type 1 bone morphogenetic protein receptor; a heterozygous c.617G>A, (p.Arg206His) mutation *de novo* is found in most FOP subjects, and a variant ACVR1 mutation occurs in the remainder (2). Individuals with FOP will appear normal at birth except for the great toe malformation. During the first decade of life, most will develop episodic, painful soft tissue swellings which mature into heterotopic bone through an endochondral process. Skeletal muscles (excepting the diaphragm, extra-ocular muscles and tongue), tendons, ligaments, fascia and aponeuroses may be affected. Progressive disability occurs as HO progresses to encasement. Most patients with FOP will be wheelchair-bound by their third decade. Death commonly results from complications of chest wall restriction.

Current management is focused on early diagnosis, avoiding injury, prompt treatment of flare-ups and functional support. A short course of prednisone is often used at the start of a flare; selective COX-2 inhibitors are also used for pain relief. Strategies for inhibiting BMP signalling include retinoic acid receptor γ agonists, small molecule inhibitors or monoclonal antibodies against ALK2, and inhibition of HIF1α/mTOR signalling.

Lessons learnt from FOP will likely be relevant to understanding non-genetic forms of HO, although the latter form through both endochondral and intramembranous ossification pathways. Therapies developed for FOP may be relevant for HO in other situations

1. Kaplan FS, et al. The medical management of fibrodysplasia ossificans progressiva: current treatment considerations. Proc Intl Clin Council FOP 2: 1-128, 2021.
2. Shore EM et al. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat Genet. 2006;38:525-7.

2

The Role of Bisphosphonates in the Management of Bone Health Issues in Childhood.

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For nearly 30 years, bisphosphonate therapy has been used in the paediatric population, but there is little high quality evidence to support its use in most bone health conditions of childhood. However, there are often few, if any, alternative therapeutic options, and therefore it is used in a wide range of clinical scenarios. The most common indication remains skeletal fragility, caused by a primary or secondary bone disorder. Osteogenesis imperfecta is the best studied of the primary bone disorders, but even in this condition there are many observational studies, but few randomized trials. Secondary bone health problems have a wide variety of antecedents, including neuromuscular disorders, malnutrition/malabsorption, and glucocorticoid therapy. In this diverse group, the evidence base is even patchier. However, for both the primary and secondary groups, bisphosphonates remain the first line therapeutic option for many patients, with the data suggesting improved bone mineral density, reduced fracture risk and reduction in bone pain.

Indications beyond skeletal fragility remain largely experimental, with observational data of its effectiveness in treating bone cysts, avascular necrosis, chronic recurrent multifocal osteomyeltitis, fibrous dysplasia, generalized arterial calcification of infancy, and hypercalcaemia.

Bisphosphonates are generally well tolerated aside from an acute phase response accompanied by hypocalcaemia with the first dose. Longer term side effects seem to be few with no strong evidence of increased risk of atypical femoral fractures, and no reported cases of osteonecrosis of the jaw in children. However, bone turnover is substantially reduced, and the long term effect of this on the developing skeleton remains unclear.

Overall, bisphosphonates remain the mainstay of treatment for skeletal fragility in children, with use also in some other bony conditions. Newer agents may lead to improved outcomes but this remains under investigation.

3

Osteoanabolic Therapy: A New Era for Osteoporosis?

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Should anabolic therapy for osteoporosis be used as first line therapy? Anabolic agents stimulate bone formation whereas anti-resorptive agents reduce bone remodelling. Australian guidelines recommend the use of an anti-resorptive agent such as bisphosphonates or denosumab as first line therapy. However, the anabolic agents teriparatide (recombinant human parathyroid hormone 1-34), and the more recently available romosozumab (anti-sclerostin antibody), result in significant reduction in fracture risk compared to bisphosphonates therapy alone. Sustained fracture risk reduction after treatment with anabolic therapy can

be achieved by consolidating bone mass accrued using subsequent anti-resorptive therapy. While current Australian treatment guidelines require prior treatment with anti-resorptive therapy, emerging data suggest the potential benefit of using anabolic agents as first-line treatment in those identified with high to very high fracture risk. Such protocols generally require subsequent anti-resorptive agent. Different combinations and sequential modalities have been studied with positive outcomes. Stratification of patients' baseline absolute fracture risk, and identifying those with high fracture risk, could potentially lead to improved long term outcomes. In this talk, evidence on the role of osteoanabolic therapy in osteoporosis, and possible new models of therapy, will be discussed.

4

The gut microbiome and bone metabolism: an update.

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Osteoporosis and its precursor osteopenia are a common metabolic bone diseases in postmenopausal women. Improvement of peak bone mass in younger age and reducing bone loss in aging are two strategies to reduce the risk for developing osteoporosis. Modulating intestinal calcium absorption by diet can contribute to improvement of bone mass, and reduction of inflammation during menopause can reduce the risk of bone loss. Prebiotics are fermented by the gut bacteria resulting in the production of organic acids which reduce the pH in the large intestine and may improve solubility of minerals thus increasing passive diffusion via the paracellular pathway. Probiotics have been reported to increase the immune system efficiency, enhance vitamin and mineral absorption as well as generate organic acids and amino acids. Bone turnover is regulated by hormones, immune cells, and the gastrointestinal system supporting mineral absorption. In addition, the intestine also produces endocrine factors such as incretins and serotonins that signal (crosstalk) to bone cells. A growing body of evidence suggests that the gut microbiota is involved in the regulation of bone metabolism but there are few studies examining how gut microbiomes in osteoporosis and osteopenia may differ from those in healthy individuals. In a pilot study the diversity, composition, and functional gene potential of the gut microbiota of healthy, osteopenic, and osteoporotic women were characterised. Both osteoporotic and osteopenic taxonomic compositions were found to be significantly different from healthy participants. Modulation of the microbiome may prove to be beneficial for the prevention of bone loss.

5

High-intensity exercise and hip bone geometry in postmenopausal women on or off bone medication: The MEDEX-OP trial

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Purpose To compare the effects of high-intensity resistance and impact training (HiRIT) to low-intensity, Pilates-based exercise on proximal femoral geometry and explore the influence of antiresorptive medication on effects.

Methods Postmenopausal women with low bone mass, on or off antiresorptive bone medications, were recruited and randomly allocated, stratified on medication intake, to eight months of twice-weekly, supervised HiRIT (Onero™) or supervised, low-intensity, Pilates-based exercise (BB, Buff Bones®). 3D hip software was used to analyse DXA scans of the non-dominant proximal femur. Outcomes included femoral neck (FN) and total hip (TH), volumetric bone mineral content (vBMC) and density (vBMD), and cortical thickness, and FN cross-sectional area, cross-sectional moment of inertia and section modulus (Z). Data were analysed using repeated measures analysis of variance.

Results Proximal femur scans of 102 women (64.7 ± 6.0 years) were examined: BB, 43; HiRIT, 37; BB-med, 11; HiRIT-med, 11. Exercise compliance did not differ between groups (83.4 ± 12.7%). HiRIT improved trabecular TH vBMC and vBMD (3.1 ± 1.1% versus -1.2 ± 1.2%, $p = 0.008$; and 1.5 ± 1.0% versus -1.6 ± 1.2%, $p = 0.042$, respectively) as well as total FN and TH vBMC (2.0 ± 0.8% versus -0.2 ± 0.7%, $p = 0.032$; and 0.7 ± 0.4% versus -0.8 ± 0.6%, $p = 0.032$, respectively), compared to losses in BB. HiRIT also increased Z while BB did not ($p = 0.035$). The combination of exercise and antiresorptive medication achieved greater improvements in multiple geometric outcomes compared to exercise alone, particularly for the HiRIT intervention.

Conclusions HiRIT provided a positive stimulus to geometric parameters of proximal femur strength while low-intensity, Pilates-based exercise was largely ineffective. Medication intake may enhance exercise effects, however, data are preliminary.

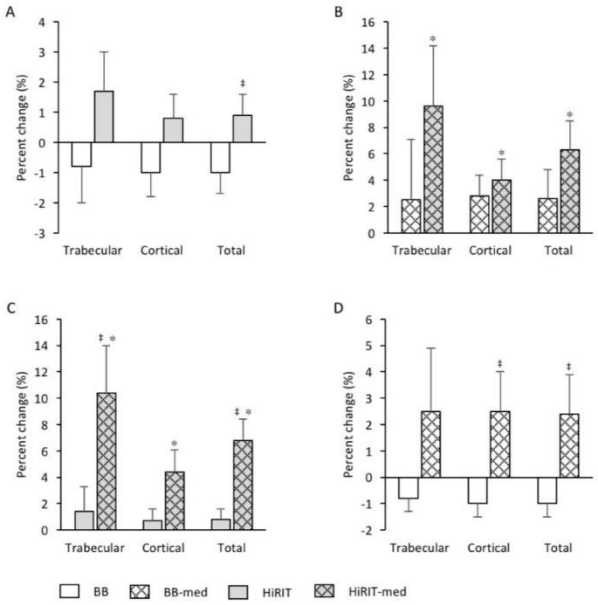

*Figure 1. Eight-month percent change (mean ± SE) in femoral neck volumetric BMC for the following subgroup comparisons: (A) BB versus HiRIT, (B) BB-med versus HiRIT-med, (C) HiRIT versus HiRIT-med, (D) BB versus BB-med. ITT data; BB n = 43, BB-med n = 11, HiRIT = 37, HiRIT-med n = 11. * Indicates within-group change from baseline ($p \leq 0.05$); ‡ Indicates between-group difference in percent change ($p \leq 0.05$). Abbreviations: BB, Buff Bones®; BB-med, Buff Bones® plus bone medications; HiRIT, high-intensity resistance and impact training; HiRIT-med, high-intensity resistance and impact training plus bone medications; ITT, intention-to-treat.*

Creatinine to cystatin C ratio: A novel biomarker of sarcopenia measures and falls risk in community-dwelling older women?

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Background: The contribution of age-related muscle impairment to falls and associated injury (e.g. fracture) is well recognised. The ratio of creatinine to cystatin C (Cr:Cyc) has been proposed as a biomarker of muscle mass, but its relationship to adverse outcomes, including falls, remain unclear. We examined the relationship between Cr:Cyc with sarcopenia measures (muscle mass and function), 5-y self-reported falls and 14.5-y fall-related hospitalizations in a prospective cohort study of 1,118 community-dwelling older women (mean±SD, age 75.2±2.7 y).

Methods: Serum Cr:Cyc, hand grip strength and timed-up-and-go (TUG) performance were assessed at baseline (1998), while dual-energy X-ray absorptiometry (DXA) derived ALM/Height $(m)^2$ was obtained in a subset of women at baseline (n=334). Incident 5-y self-reported falls and 14.5-year fall-related hospitalizations from linked health records were examined.

Results: In a multivariable-adjusted model, each SD decrease in Cr:Cyc was associated with reduced grip strength (β=-0.11, p<0.001) and ALM/Height^2 (β=-0.13, p=0.002), but not TUG (β=0.01, p=0.086). Women with the lowest Cr:Cyc (Quartile [Q] 1) had 6.6% (1.4 kg) weaker grip strength and 3.3% (0.2 kg/m^2) lower ALM/Height^2 compared to women in Q4 (both p<0.05). Overall, 329 women reported an incident fall over 5 years, and 439 fall-related hospitalizations were recorded over 14.5 years. Women in Q1 of Cr:Cyc had a greater relative hazard for falls (HR 1.50 95%CI 1.11-2.01) and fall-related hospitalizations (HR 1.48 95%CI 1.14-1.91) compared to Q4 in the multivariable-adjusted model (*Figure 1*). Results remained unchanged when grip strength was included in the multivariable-adjusted model.

Conclusion: The use of Cr:Cyc may represent a viable muscle biomarker to help clinicians identify individuals at risk of falls who may benefit from primary prevention programs (e.g. diet and exercise). Future work should seek to confirm the utility and cut-points of Cr:Cyc as a biomarker contributing to sarcopenia diagnosis and management.

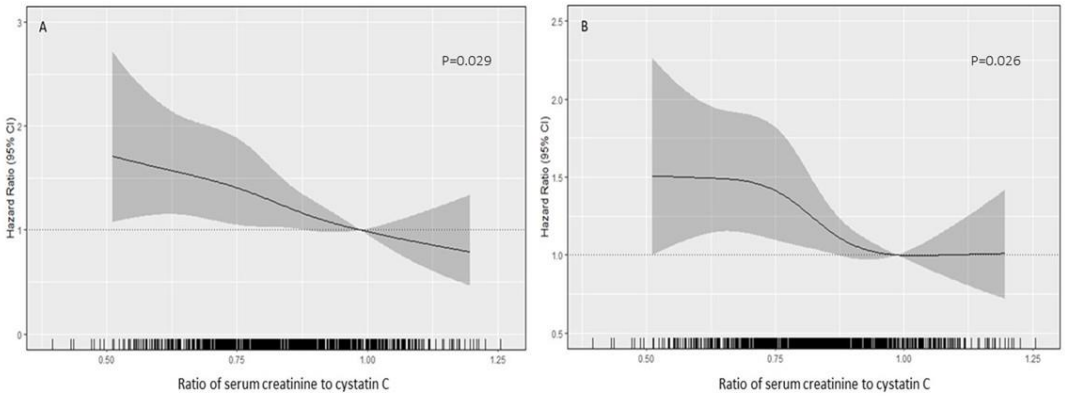

Figure 1. Hazard ratios from Cox proportional hazards model with restricted cubic spline curves describing the association between serum creatinine to cystatin C (Cr:Cyc) ratio and (A) 5 y self-reported incident falls, (B) 14.5-year fall-related hospitalizations. Hazard ratios are based on models adjusted for age, calcium treatment, BMI, smoking history, diabetes status, prevalent atherosclerotic vascular disease, physical activity, prevalent falls, and are comparing the specific level of Cr:Cyc (horizontal axis) to the median value for women in the highest quartile (0.99). Shading represents 95% confidence intervals. The rug plot along the bottom of each graph depicts each observation.

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Associations between Vitamin D Receptor polymorphism, Childhood Fracture Risk and Maternal Vitamin D Levels in utero: The Vitamin D in Pregnancy Study

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Background:

Fractures are a common childhood injury. Polymorphisms in the vitamin D receptor gene (VDR) are associated with fracture risk in adults however, studies in children are limited. Previous work in the Vitamin D in Pregnancy (VIP) cohort found that maternal 25(OH)D concentration was associated with decreased fracture risk in boys at early gestation. This study aimed to determine the association between the child's VDR genotype and their childhood fracture risk and whether their VDR genotype modifies the effect of maternal vitamin D *in utero*.

Methods:

At birth, 402 mother-child pairs from the VIP study had at least one serum 25(OH)D measure during pregnancy and 341 children had deoxyribonucleic acid extracted from a blood spot on their Guthrie card to determine their VDR genotype. Offspring fractures were identified through examining radiological reports from birth (2002-2004) until December 31st 2012. Multivariable Cox regression models were developed to examine associations and interaction terms were tested to determine if there was an interaction between VDR and maternal vitamin D concentrations.

Results:

In total, 341 mother-child pairs had complete information. There was no significant association between the offspring's VDR genotype (*Bsm*I, *Apa*I, *Taq*I and *Fok*I) and their fracture risk. There was evidence of the offspring's *Fok*I genotype modifying the effect of maternal vitamin D in boys at early gestation (p=0.025). The ff genotype in boys was associated with a decreased fracture risk at recruitment (adjusted HR 0.78; 95% CI 0.64-0.96; p=0.021), while the FF and Ff genotypes were associated with increased fracture risk in girls (adjusted HR 1.02; 95% CI 1.00-1.03; p=0.042).

Conclusion:

Within this cohort, there is sexual dimorphism in the effects of the offspring's VDR, specifically with the different *Fok*I alleles between sexes mediating the action of maternal vitamin D *in utero* in opposing directions.

8

Bisphosphonates and bone mineral density in patients on haemodialysis and renal transplant: a 15-year single-centre experience

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Chronic Kidney Disease Stage 5D (CKD-5D) imparts a 4-fold increase in minimal trauma fracture with a substantial increase in mortality following hip fracture. Bone disease in CKD is complex, characterised by abnormal levels of PTH, calcium, phosphate, ALP, and vitamin D, manifesting as a condition known as CKD-Mineral and Bone Disorder (CKD-MBD). While bisphosphonates (BP) are the gold-standard in the management of osteoporosis, their therapeutic role when end-stage renal function and bone disease co-exist remains unclear. This 15-year retrospective cohort study examines the long-term use of BPs in 148 CKD patients receiving haemodialysis and renal transplant in a tertiary centre in Sydney, Australia. In multivariate regression adjusting for age, baseline BMD and fracture incidence, BPs increased bone density in renal transplant recipients over a mean treatment period of 3.5 years (net annual BMD gain of 0.039 g/cm^2, p=0.005). No such benefit was seen in BMD in subjects on haemodialysis and treated with BPs (net annual BMD gain of 0.008 g/cm^2, p=0.62). BP therapy did not result in significant changes in biochemical parameters (ALP, PTH, and phosphate) and there was no evidence that BPs resulted in adynamic bone disease in CKD over the 15-yr period. Whilst a significant decline in eGFR in haemodialysis patients on BPs was noted, no such effect was seen in transplant recipients. BPs are generally safe and effective in CKD5, mitigating bone loss in kidney transplant recipients without increasing the risk of adynamic bone disease or transplant failure over the 15-year study period.

9

Kinetic reconstruction of the cancellous (Cn) and endocortical (Ec) remodeling unit reveals a net positive bone balance after 12 months (M12) of treatment with romosozumab (Romo)

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Purpose:

To assess the effect of Romo on bone formation and resorption at the level of the basic multicellular unit (BMU) and determine the integral effects on BMU bone balance (BB).

Methods:

Transiliac biopsy sections from FRAME (NCT01575834) were obtained after M12 of Romo or placebo (Pbo) treatment; histomorphometric parameters were measured on Cn and Ec envelopes. Erosion depth (E.De; classified by the predominate resorptive cell type), osteoid thickness (O.Th), and complete and incomplete wall thickness (W.Th) were based on counting of lamellae in polarized light. To assess the formative site at M12, O.Th/mineralized (Md) W.Th at osteoid surfaces were classified using a 4-sector system; the sector containing the nearly completed formative sites provided the reconstructed W.Th. Kinetics of remodeling phases were reconstructed according to Steiniche (*Bone* 1992).

Results:

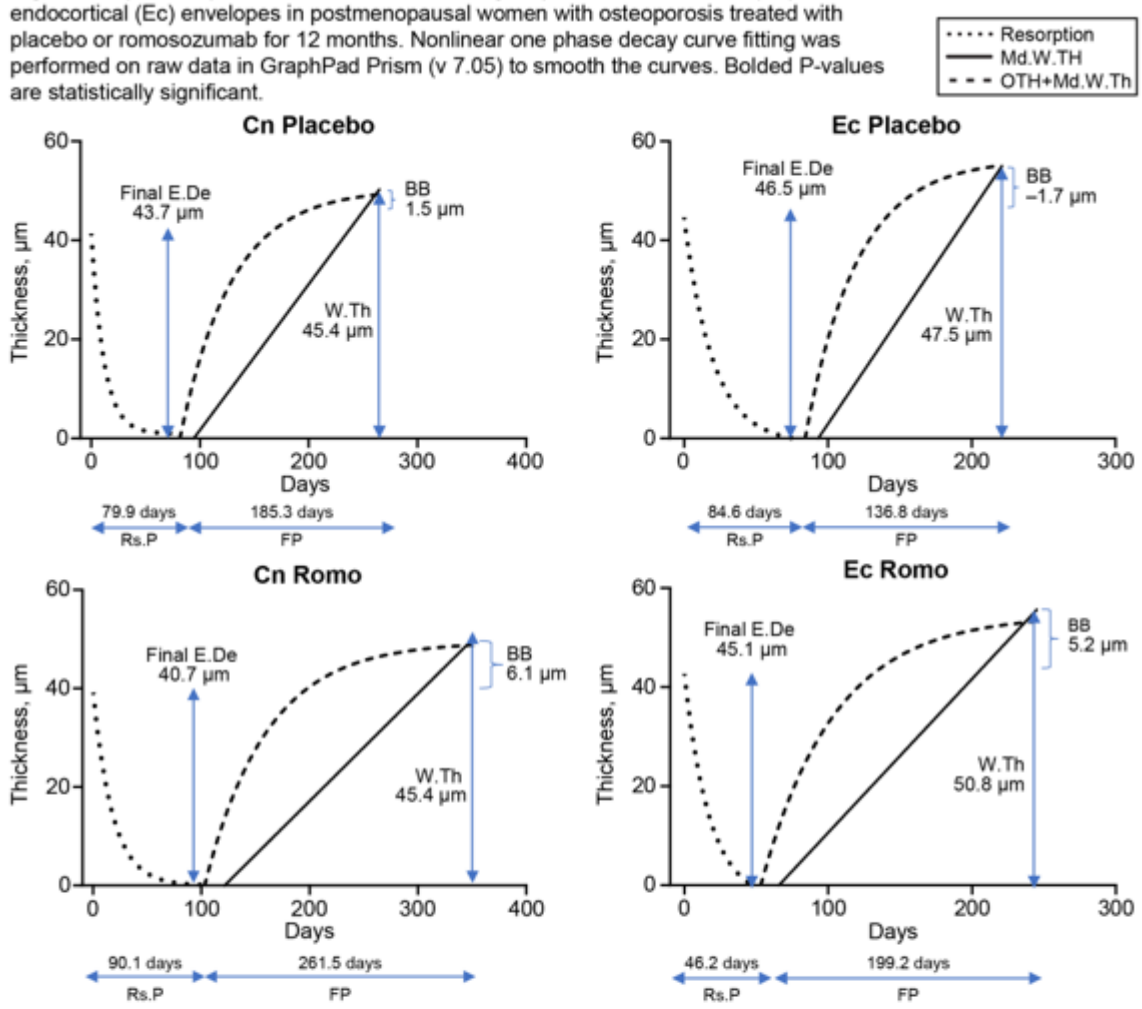
On Cn envelope, Romo resulted in a net positive BB compared with Pbo primarily due to sustained reduction in resorptive cell activity throughout 12 months, resulting in a significant reduction in final (Pre-Ob) E.De. On Ec envelope, Romo also resulted in net positive BB, not due to effects on resorption but due to positive effects on the formative site. Consequently, Romo significantly increased W.Th in bone packets that had completed by M12. This effect was not sustained in actively forming packets at the end of treatment, suggesting these positive effects on bone formation at the BMU occurred earlier in treatment.

Conclusion:

After 12 months of Romo, a positive BB at the level of individual BMUs was evident on Cn and Ec envelopes, predominantly due to a net decrease in resorptive cell activity in Cn bone and a net increase in osteoblastic function in Ec bone. These effects likely contribute to the progressive increase in bone mass and microarchitectural improvements with Romo across 12 months of

treatment.

Figure. Graphical representation of the remodeling sequence on cancellous (Cn) and endocortical (Ec) envelopes in postmenopausal women with osteoporosis treated with placebo or romosozumab for 12 months. Nonlinear one phase decay curve fitting was performed on raw data in GraphPad Prism (v 7.05) to smooth the curves. Bolded P-values are statistically significant.



BB = bone balance; FP = formative phase; Md.W.Th = mineralized wall thickness; OTh = osteoid thickness; Final E.De = pre-osteoblast (final) erosion depth; Rs.P = resorptive phase; W.Th = wall thickness

	Placebo N = 31	Romosozumab N = 39	P-value
Remodeling Parameter, µm, median (Q1, Q3)			
Cancellous envelope			
Osteoclast erosion depth	18.1 (14.5, 22.4)	15.4 (11.1, 18.4)	**0.027**
Mononuclear cell erosion depth	33.6 (27.7, 36.9)	29.3 (22.4, 34.1)	0.067
Final erosion depth	43.7 (40.0, 49.0)	40.7 (36.7, 46.2)	**0.05**
Wall thickness	45.4 (41.5, 48.8)	45.4 (41.6, 50.6)	0.57
Reconstructed wall thickness	51.2 (46.2, 55.5)	51.2 (46.7, 57.2)	0.64
Bone balance	1.5 (–6.1, 6.1)	6.1 (1.5, 9.0)	**0.012**
Endocortical envelope			
Osteoclast erosion depth	19.2 (14.9, 23.0)	12.8 (9.6, 17.1)	**<0.001**
Mononuclear cell erosion depth	33.1 (28.6, 40.9)	26.7 (21.5, 30.7)	**<0.001**
Final erosion depth	46.5 (43.3, 55.8)	45.1 (39.2, 52.9)	0.33
Wall thickness	47.5 (41.9, 52.1)	50.8 (45.4, 53.3)	**0.037**
Reconstructed wall thickness	56.0 (48.9, 63.1)	56.5 (49.9, 62.1)	0.84
Bone balance	–1.7 (–6.6, 3.4)	5.2 (–2.0, 11.9)	**0.02**

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Discovery to Industry

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Discovery to the Clinic

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Discovery to Policy

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13

Elucidating human skeletal development using single-cell analyses with a model of endochondral bone formation using human pluripotent stem cells

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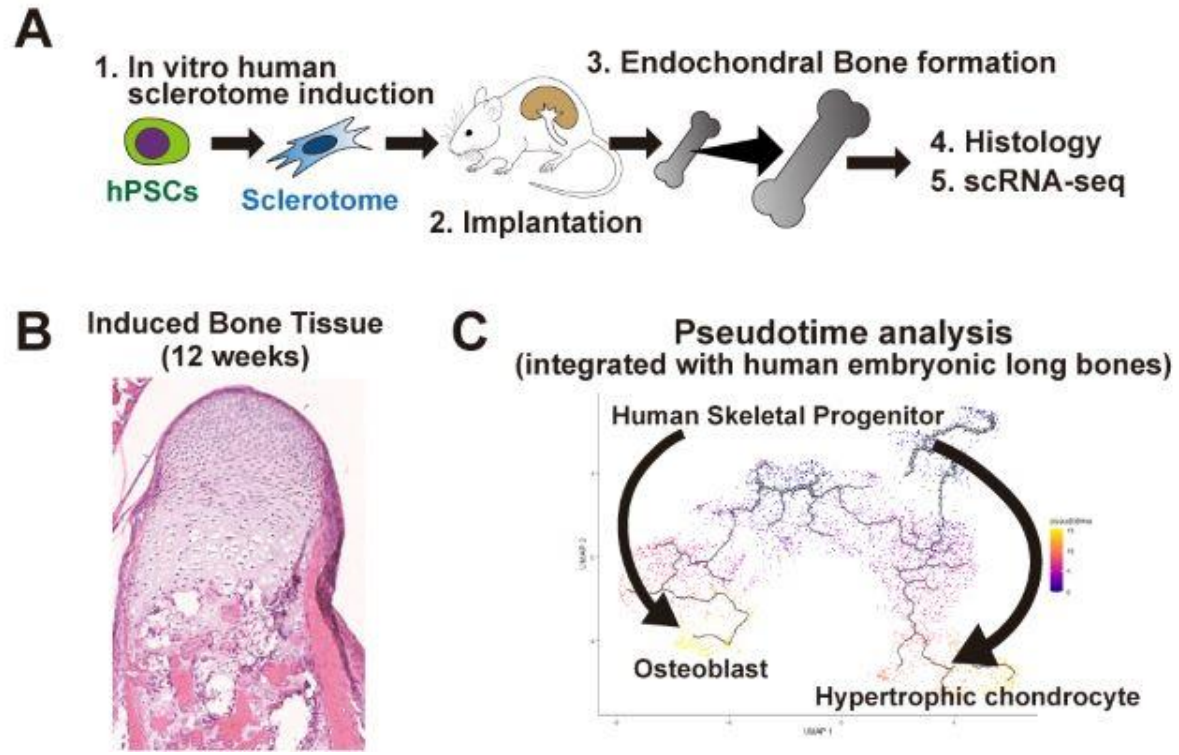
Modeling human skeletal development is an essential step in elucidating detailed mechanisms underlying this process. Although human pluripotent stem cells (hPSCs) can differentiate into any embryonic cell type, it is challenging to recapitulate 3D bone tissues composed of multiple cell types. To generate human bone tissues, we induced the in vitro differentiation of hPSCs into sclerotome and implanted them beneath the renal capsules of immunodeficient mice. RNA-seq analysis demonstrated cell type-specific gene expression, indicating a stepwise differentiation of hPSCs into the sclerotome. In vivo micro-CT images obtained after implantation showed the growth of mineralized tissues over time. Histological analyses revealed endochondral bone-like structures with specific marker expression patterns: columnar structure of chondrocytes, bone collar, and bone marrow. The induced bone tissues were then analyzed by single-cell RNA-seq, and the obtained data were integrated with the publicly available gene expression profiles of human embryonic long bones at 8 weeks post conception (He J. et al., Cell Res. 2021). Clustering analysis identified multiple skeletal cell types with distinct gene expression signatures; the gene expression profiles of the hPSC-derived bone tissues overlapped with those of the embryonic long bones. Pseudotime analysis predicted a bifurcating trajectory from skeletal progenitors to osteoblasts or chondrocytes. By integrating differential gene expression analysis, gene regulatory network analysis, and ligand-receptor analysis, we extracted novel transcriptional regulators that may play important roles in human osteogenesis. In situ hybridization of the hPSC-derived bone tissues showed a partial co-expression of the identified regulators with *RUNX2* and *SP7*. The knockdown of these regulators downregulated osteoblast marker genes in a human osteosarcoma cell line (Saos2), indicating the involvement of these regulators in human osteogenesis. Collectively, our bone induction method may provide a valuable model of human endochondral bone formation, enabling us to investigate hard-to-access human skeletal development.



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EphrinB2 limits mineralisation in osteocytes in an mTOR-dependent manner by downregulating the lysosomal master transcriptional regulator TFEB

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Lysosomes are acidic vesicles that degrade cytoplasmic contents, and while they are essential for osteoclast-mediated bone resorption, their function in osteocytes is unknown. We recently observed that EphrinB2 (*Efnb2*) deletion in osteocytes leads to hypermineralised, fragile bones and reduced osteocyte lysosome content. Osteocyte-mediated mineralisation is limited by RhoA-associated protein kinase, downstream of the mammalian Target Of Rapamycin (mTOR) signalling pathway. Lysosome biogenesis is controlled by Transcription Factor EB (TFEB), a substrate of mTOR. Here, we sought to define whether EphrinB2 limits lysosome numbers and mineralisation through an mTOR-dependent molecular pathway.

To determine the cause of lysosome deficiency, we measured TFEB in *Efnb2*-deficient Ocy454 osteocyte-like cells and found that TFEB mRNA and protein levels were reduced, by 50% and 66%, respectively. Proteomic analysis confirmed that multiple known TFEB targets, including lysosomal hydrolases, membrane and acidification proteins were significantly downregulated in *Efnb2*-deficient osteocytes.

We next defined the upstream and downstream targets of mTOR signalling modified by *Efnb2* deficiency in osteocytes. Western blotting revealed no difference in upstream AMPK activity (pT172), but AKT activity (pT308) was doubled in *Efnb2*-knockdown cells compared to control. mTOR has two downstream signalling complexes, mTORC1 and mTORC2. While we found no change in active RhoA, an mTORC2 target, in hypermineralising *Efnb2*-knockdown cells compared to controls, mTORC1 activation (p70S6K (pT389)) was doubled. Furthermore, when we inhibited mTORC1 activity with rapamycin, this reduced mineral deposition by ~95% in wildtype Ocy454 cells. This suggests that EphrinB2 loss elevates AKT activity, leading to higher mTORC1 activity and greater mineralisation.

These data indicates that EphrinB2 may restrain mineralisation by increasing TFEB activity and lysosome numbers in osteocytes through limiting AKT and mTORC1 activity. This suggests a novel role for TFEB and lysosomes in osteocyte function, specifically in the control of mineralisation and bone strength.

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Determining the role of maternal epigenetic inheritance on bone development and disease

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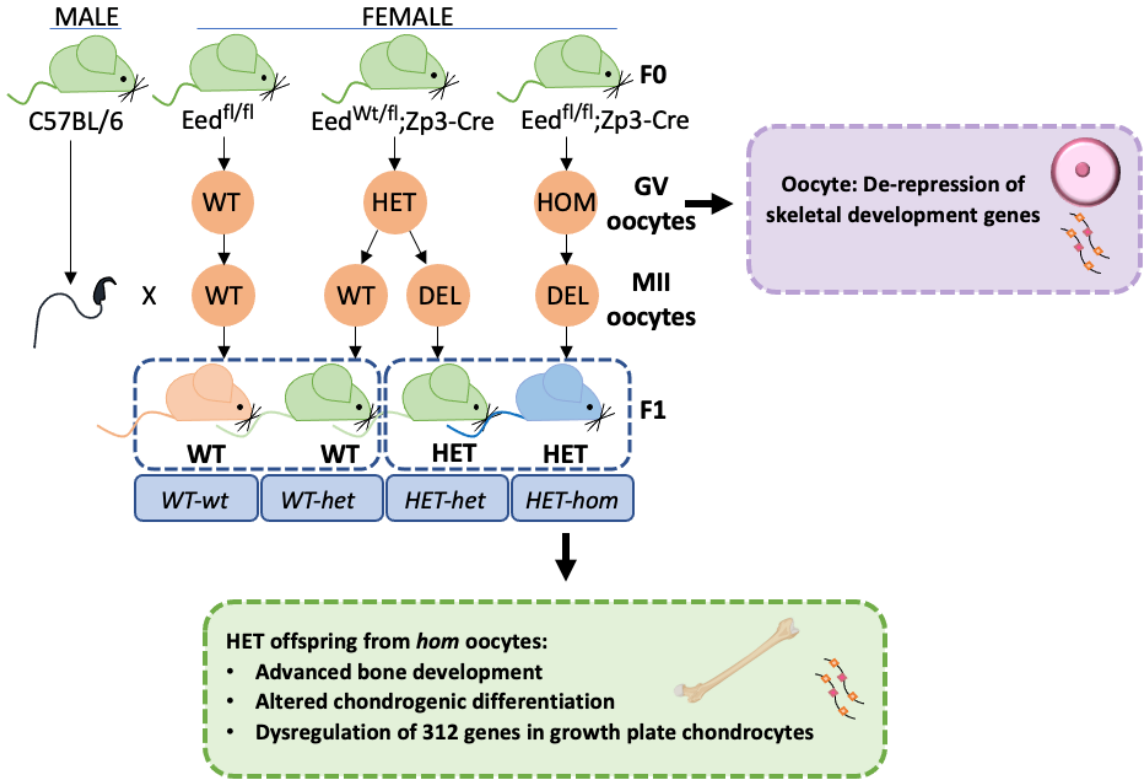
Epigenetic programming in the germline is considered to affect development in offspring, but the mechanisms are poorly understood. Embryonic Ectoderm Development (EED) is essential for Polycomb Repressive Complex 2 (PRC2) function, which epigenetically regulates developmental genes in bone. PRC2 is a known regulator of bone stem cell differentiation and is implicated in maintenance of adult bone health. *De novo* germline mutations in human EED result in Cohen-Gibson syndrome, characterized by overgrowth, accelerated bone aging and skeletal defects. However, EED's potential to alter oocyte epigenetic programming and consequent offspring development is poorly understood.

To determine the role of EED in oocyte programming, we developed a mouse model in which *Eed* is specifically deleted in growing oocytes. This model facilitates the production of genetically identical heterozygous offspring from oocytes with differences in their epigenetic heritage. We predicted that offspring from oocytes lacking EED would have impaired bone development, as well as compromised bone maintenance and repair.

Loss of EED in oocytes resulted in the de-repression of 244 genes (FDR<0.05) that were primarily associated with fetal development, including bone formation. MicroCT analyses demonstrated that postnatal day 3 offspring from oocytes lacking EED exhibited greater bone mineral density, increased mineralised bone length ($p<0.05$, n=8-10) and bone width ($p<0.0005$, n=8-10). Histological and RNAseq analyses revealed that loss of EED in oocytes resulted in an increased hypertrophic zone ($p<0.05$, n=8-12) and dysregulation of 312 genes (FDR<0.05) in E17.5 femoral growth plates, indicative of abnormal transcriptional regulation and chondrocyte differentiation in heterozygous experimental offspring, compared to genetically identical heterozygous controls.

Together, these data strongly suggest that altered EED-dependent oocyte programming results in postnatal overgrowth and altered bone development, including similarities to skeletal defects associated with Cohen-Gibson syndrome. This model will be used to identify how inherited epigenetic information controls early life and long-term skeletal development and health.



Vitamin D supplementation and exercise for improving physical function, body composition and metabolic health in overweight or obese older adults with vitamin D deficiency: a randomised, double-blind, placebo-controlled trial

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Background: Vitamin D supplementation may have non-skeletal health benefits and enhance exercise responsiveness, particularly in those with low vitamin D concentrations. We investigated whether vitamin D supplementation taken prior to, or during, a 12-week exercise program improves metabolic health, body composition or physical function in overweight and obese older adults with vitamin D deficiency.

Methods: Fifty overweight and obese older adults (mean±SD age: 60±6 years; BMI 30.6±5.7) with vitamin D deficiency (25-hydroxyvitamin D [25(OH)D] <50nmol/L) were recruited. Participants were randomly allocated to receive either vitamin D_3 (4000 IU/day) or matching placebo for six months. Between months 3-6, all participants completed a 12-week multi-modal exercise program (aerobic and resistance exercise) at a frequency of three days per week (one supervised and two home-based sessions) while continuing with vitamin D/placebo. Mean changes in biochemical parameters, body composition and physical function at three and six months were compared between groups.

Results: At three months, vitamin D supplementation increased 25(OH)D levels (placebo = 2.5±14.7nmol/L; treatment = 43.4±18.4nmol/L; P<0.001) and reduced stair climb times (placebo = 0.3±1.0sec; treatment = -0.2±1.0sec; P=0.046). At six months, vitamin D supplementation combined with multi-modal exercise reduced waist circumference (placebo = 1.3±7.3cm; treatment = -3.0±6.1cm; P=0.022) and decreased waist-to-hip ratio (placebo = 0.01 ± 0.05; treatment = -0.03 ± 0.05; P=0.005). Vitamin D supplementation had no effect on gait speed (primary outcome) or any other biochemical, body composition or physical function parameters when taken alone, or in combination with exercise.

Conclusion: Vitamin D supplementation increased 25(OH)D levels and augmented waist circumference losses following a multi-modal exercise program in overweight and obese older adults with vitamin D deficiency. Vitamin D supplementation alone also reduced stair climb times. Future studies should focus on individuals with moderate or severe vitamin D deficiency, as these individuals might experience greater therapeutic benefits.

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Trabecular bone score is associated with prospective fracture and refracture risk in Australian adults

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Background

Trabecular bone score (TBS) applies an algorithm to lumbar spine (LS) dual x-ray absorptiometry (DXA) scans to assess trabecular microarchitecture. TBS may improve the assessment of fracture risk, complementary to bone mineral density (BMD), and may be useful for identifying those likely to refracture. This study aimed to investigate associations between TBS and incident fracture, including after a prior fracture.

Methods

Men (n=894) and women (n=681) aged 24-98yr from the Geelong Osteoporosis Study were included. LSBMD L2-L4 (Lunar Prodigy) and TBS L1-L4 (TBS iNsight V2.2) were calculated and incident fractures identified radiologically (any low trauma fracture; major osteoporotic fracture [MOF; hip, spine, proximal humerus, wrist]). Cox-proportional hazards modelling (from date of DXA scan to first fracture, death, or 31/12/2016) were used to explore associations between lower TBS and fracture in the whole cohort and in a subset of participants with prior fracture (<10yr before TBS measurement), adjusting for age, height, weight, smoking, mobility, alcohol consumption, falls and medication use.

Results

Fifty-five participants reported an incident fracture (17 clinical spine, 4 wrist, 9 hip, 2 proximal humerus, 7 distal tibia/fibula, 6 tarsals/metatarsals, 6 rib, 3 metacarpal, 2 pelvis and femur, 1 each proximal tibia/fibula, elbow, carpal, scapula, patella), at a rate of 5.8/1000 person-years (95%CI:4.4-7.5). Of 151 participants with a prior fracture, 13 reported an incident fracture (9 MOFs), incidence 16.4/1000 person-years (95%CI:9.5-28.2).

Lower TBS was associated with increased risk of fracture (unadjusted HR=1.30, 95%CI:1.09-1.56) and MOF (HR=1.56, 95%CI:1.24-1.97); adjustment for confounders and LSBMD attenuated results (HR=1.04, 95%CI:0.86-1.27 and HR=1.21, 95%CI:0.93-1.57 respectively). Among participants with prior fracture, results for refracture (HR=1.59, 95%CI:1.11-2.27) were attenuated with adjustment (HR=1.39, 95%CI:0.91-2.12); results for MOFs were sustained (unadjusted HR=1.73, 95%CI:1.15-2.59, adjusted HR=2.42, 95%CI:1.09-5.36).

Conclusion

Lower TBS was associated with incident fracture and refracture. The relationship between TBS and MOF refractures was independent of LSBMD.

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Muscle strength and physical performance are associated with risk of post fracture mortality but not subsequent fracture

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Background: Muscle strength and physical performance measurements are associated with incident fractures and mortality. However, their impact on post fracture outcomes is not clear.

Objective: To assess the association between muscle strength and performance with subsequent fracture and mortality after a fracture.

Methods: The study included 830 MrOS USA participants with low-trauma index fracture who had muscle strength and physical performance assessments within 5 years prior to the index fracture. Mixed effects models were used to estimate the annual change in muscle strength and performance following index fracture. Index value and decline of each test were examined as predictors of subsequent fracture and mortality using Cox proportional hazards models adjusted for age, FNBMD, prior fractures, falls, BMI, index fracture type, lifestyle factors, and comorbidities.

Results: Median follow-up from index fracture to mortality was 5.1(IQR: 1.8-9.6) years and to subsequent clinical fracture was 3.7 years (1.3-8.1). During follow-up, 536 (65%) men died and 201 (24%) men had a subsequent fracture. Index strength and performance were independently associated with mortality risk; HRs per SD change for lower grip strength, lower gait speed, and greater sit-to-stand time were 1.12(95%CI: 1.01-1.25), 1.14(1.02-1.27), and 1.08(0.97-1.21), respectively. The annual decline in these tests was also associated with increased mortality risk, independent of the baseline value and other confounding effects (HR per SD of the change: 1.15(95% CI: 1.01-1.33), 1.38(1.13-1.68), 1.28(1.07-1.54), respectively). Men who were unable to complete one or multiple tests had even greater risk of mortality. However, muscle strength and performance were not associated with subsequent fractures.

Conclusion: Both lower muscle strength and physical performance measured prior to index fracture as well as their decline were associated with post-fracture mortality but not subsequent fracture risk. However, it remains to be seen whether improvement in these parameters can reduce mortality risk after incident fracture.

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Is recovery of quality of life post-fracture associated with 5-year mortality? The Australian arm of the International Costs and Utilities Related to Osteoporotic fractures Study (AusICUROS)

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10. The Institute for Mental and Physical Health and Clinical Translation (IMPACT), Deakin University, Melbourne, VIC, Australia

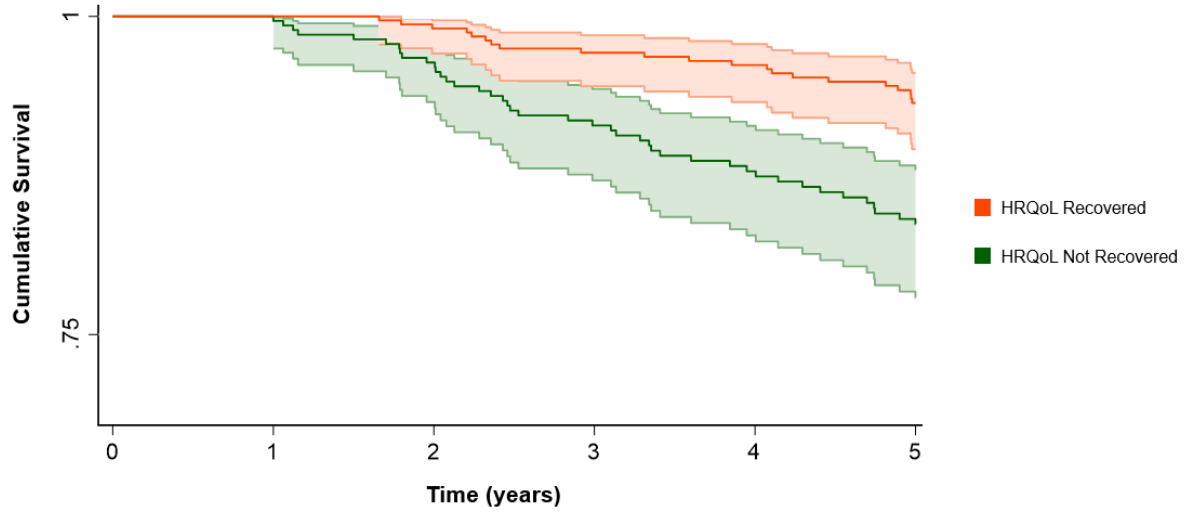
Aim: While evidence that suggests fractures are associated with increased mortality and decreased health-related quality of life (HRQoL), it is unknown if these outcomes are correlated. This study aimed to determine whether recovery of HRQoL 12-months post-fracture is associated with lower 5-year all-cause mortality.

Methods: This prospective study included 524 older adults (mean age: 70.2 years; % female: 79.2) with a fracture (150 hip, 261 wrist, 61 vertebral, 52 humerus) recruited from eight study centers across Australia. HRQoL was assessed using the EQ-5D-3L at baseline (including recall of HRQoL prior to fracture) and at 12-months post-fracture. HRQoL recovery was calculated as the difference between EQ-5D-3L utility scores at pre-fracture and 12-months. All-cause mortality was ascertained through linkage with the Australian National Death Index. Overall survival was compared between the two HRQoL groups (recovered vs. not recovered) using a two-sided log-rank test. Cox proportional hazards models were used to assess the association between mortality and HRQoL recovery.

Results: Overall, 279 participants (53.2%) recovered to their pre-fracture HRQoL at 12-month follow-up and there were 70 deaths (13.4%) during the 5-years post-fracture. Mortality rate was highest in hip fracture participants (24.7%), followed by vertebral (16.4%), humeral (13.5%) and wrist fracture participants (6.1%). After adjustment for age, sex, pre-fracture HRQoL, and skeletal site of the fracture, mortality risk was lower in participants who recovered their pre-fracture HRQoL at 12-months compared to those who did not (HR=0.56, 95% CI: 0.33-0.96, p=0.034; see figure).

Conclusion: This study provides evidence that HRQoL recovery post-fracture is associated with improved long-term survival in older adults, and also highlights important contributions of a variety of factors that predict long-term mortality post-fracture. Current

post-fracture interventions known to improve HRQoL may have the potential to prevent deaths in older adults following fragility fracture.



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Endogenous glucocorticoid signalling plays an important role in surgically induced murine osteoarthritis which is accelerated by chronic disruption of circadian rhythm

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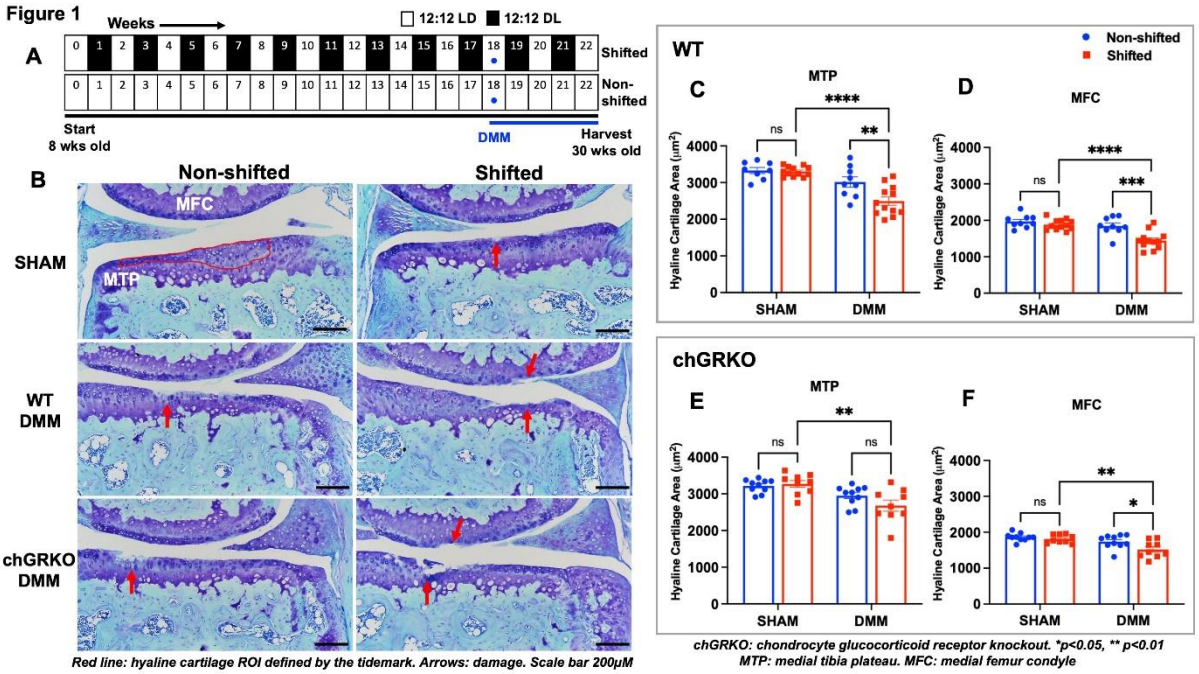
Recent evidence suggests chronic disruption of circadian rhythms such as shift work increases the risk of osteoarthritis (OA). Endogenous glucocorticoid secretion follows a diurnal rhythm and is known to regulate circadian clock expression. We aimed to investigate if endogenous glucocorticoid signalling mediates the effects of environmental circadian rhythm disruption at early stages of OA development using a surgically-induced model of OA in wild-type (WT) and tamoxifen-inducible chondrocyte glucocorticoid receptor knockout (chGRKO) mice.

Eight-week-old WT and chGRKO littermates were maintained for 22 weeks on either a normal 12 hour:12 hour light-dark cycle (non-shifted) or exposed to weekly alternating 12 hour phase shifts (shifted, Fig.1A). Four weeks prior to harvest, all mice underwent surgical destabilization of the medial meniscus (DMM) with the contralateral limb used as SHAM control (n=8-12/group).

Gene expression analysis confirmed that compared to non-shifted mice, clock genes were significantly disrupted in the long bones of shifted mice. Histological scoring showed a trend towards worse cartilage damage in shifted WT-DMM compared to non-shifted WT-DMM mice. Histomorphometry revealed that the medial tibial (MTP) hyaline cartilage area was reduced in shifted WT-DMM compared to non-shifted WT-DMM mice (2,494 vs. 3,015μm^2 p=0.0036; Fig.1B,C). At the medial femur condyle (MFC), hyaline cartilage area was also reduced in shifted WT-DMM compared to non-shifted WT-DMM mice (1,446 vs. 1,846μm^2 p=0.0004; Fig.1D). In chGRKO mice, hyaline cartilage area was only reduced at the MFC in shifted compared to non-shifted DMM mice (1,519 vs. 1,740μm^2 p=0.0371; Fig.1E,F). Both micro-CT and histomorphometry analysis revealed that only shifted WT-DMM mice developed subchondral bone sclerosis suggesting these animals developed OA at an accelerated rate compared to all other groups at this early timepoint.

Our results indicate that chronic disruption of circadian rhythms may accelerate OA, mediated via changes in tissue-specific (chrondrocytic) glucocorticoid signalling.



Red line: hyaline cartilage ROI defined by the tidemark. Arrows: damage. Scale bar 200µM

*chGRKO: chondrocyte glucocorticoid receptor knockout. *p<0.05, ** p<0.01*
MTP: medial tibia plateau. MFC: medial femur condyle

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Some Things Fishy – Insights for Reproductive Biologists from Fish

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Reproduction is essential to the evolutionary persistence of all life. Failure to reproduce, or producing offspring that are unlikely to survive to reproduce themselves, increases the risk that the genetic legacy an individual carries will be consigned to the evolutionary scrap heap. Given these life or death stakes, it is unsurprising that organisms have developed an astonishing array of approaches to increase their reproductive success across their lifetime, find and identify appropriate mates, and ensure they have a competitive advantage when they have mating opportunities. In fishes, the focus of much of my research, the solutions employed to address these challenges span sex-change, pre- and post-mating mate choice, and a range of reproductive strategies and tactics. Here, I will highlight work from my lab that is revealing the genetic underpinnings of sex change in fish, cover work that ultimately identified a new form of post-mating sexual selection, and finish up with some new insights on the genetic, epigenetic and environmental factors that influence sperm function and male reproductive success.

22

Tackling Major Complications of Pregnancy: Novel Approaches to Understand and Treat the Underlying Pathophysiology.

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The placenta is a complex organ, essential for all mammalian reproduction. Despite its critical importance, our understanding of the processes that ensure successful placentation are limited. My work has aimed to advance our knowledge of these processes controlling embryo implantation, placentation, and maternal vascular adaptation in pregnancy.

More recently my research program has focused on the development of novel therapeutics and innovative delivery strategies to improve our ability to regulate these processes to enhance pregnancy success, as well as mediate the pathological processes that occur in major complications of pregnancy. Especially preeclampsia, a serious complication of pregnancy, responsible for >70,000 maternal deaths worldwide and far greater perinatal loss. *There are currently no efficacious treatments to halt disease progression* other than delivery. A therapeutic advance is urgently needed.

The key pathophysiological stages in preeclampsia include 1) placental damage and oxidative stress, 2) elevated anti-angiogenic and pro-inflammatory factors and 3) endothelial and vascular dysfunction.

Our team has developed novel preclinical screening approaches, utilising primary human tissue models and mouse models of disease to test innovative therapeutic strategies, which include examining new drugs and innovative delivery methods.

We have now generated exciting preclinical findings repurposing drugs with good safety profiles and targeted nanoparticle therapeutic delivery directly to the placenta to mitigate the pathological progression of this syndrome.

Importantly the novel strategies we are developing for major complications of pregnancy are focused towards clinical translation and offer exciting possibilities for the future management of obstetric diseases.

23

Complications Are Now the Key Focus of Diabetes Management

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For most of the last hundred years, the management of diabetes has been focused on achieving and maintaining optimal control of plasma glucose levels. Studies like UKPDS (2000) clearly demonstrated that the lower the cumulative exposure to elevated glucose levels, the lower the risk of diabetic complications, including eye, kidney, foot and heart disease. However, once complications develop, glucose control and standard of care is not enough, and other interventions are needed to change hard outcomes. In particular, recent large cardiovascular outcome trials have demonstrated that SGLT2 inhibitors are able to reduce major acute cardiovascular events (MACE), hospitalisation for heart failure and the development and progression of impaired kidney function. Some studies have also demonstrated benefits on patient survival. In addition, trials with some GLP-1 receptor agonists have also demonstrated reductions in MACE outcomes. This new evidence has resulted in changes in drug indications as well as global guidelines for diabetes management, which now recommend prioritising these agents in patients with or at high risk for complications. And in the future, these agents will likely become a foundational part of modern diabetes management, as the cost implications of preventing of diabetic complications far outweighs the cost of medication to prevent them.

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RAGE as a Novel Therapeutic Pathway to the Prevention of Type 1 Diabetes

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Type 1 diabetes (T1D) is the most common incurable, early onset, chronic disease. Disease etiology includes autoimmunity against the β cells accompanied by progressive abnormalities in insulin secretion and action ultimately requiring life-long management with exogenous insulin. Preservation of functional pancreatic β cells reduces complications risk and improves mental health, thus new treatments preventing disease onset and/or prolonging β cell function and survival, would reduce the burden of T1D in both Australia and globally.

The receptor for advanced glycation end products (RAGE), is an important protein for host-pathogen defense and is present on cell types implicated in the development of T1D including T cells, macrophages, dendritic cells and pancreatic islet cells. RAGE also has a shortened isoform, soluble RAGE (sRAGE) that inhibits cell membranous RAGE signal transduction by competitive binding of RAGE ligands. Changes in RAGE expression are associated with T1D risk and T cells from these at-risk individuals who progress to T1D, have greater RAGE expression, which enhances cytokine production and survival. Increases in circulating RAGE ligands, AGEs, are also an independent risk factors and improve T1D risk prediction in UK population and twin-based studies and impact beta cell function. Blockade of RAGE ligands prediabetes reduces T1D onset in murine models. Single nucleotide polymorphisms in the RAGE gene (*AGER*) also decrease circulating soluble RAGE (sRAGE) concentrations and increases the risk for T1D. This decline in circulating sRAGE also coincides with the onset of autoimmunity, seen as seropositivity for autoantibodies against islet auto-antigens in at-risk individuals. Targeting T1D using soluble RAGE slows the development of T1D incidence and reprograms the immune system eliciting its effects via T regulatory (Treg) cells. The contribution of AGE-RAGE pathway to T1D and how it may be targeted, forms the basis of this presentation.

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Testosterone and Type 2 diabetes in Men. The Chicken, the Egg, or the Bees' Knees?

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Men with obesity and insulin resistance have lower serum testosterone (T) concentrations than age matched healthy men. The low serum T is an independent risk factor for incident type 2 diabetes (T2D) in these men.

In order to determine whether T treatment for 2 years decreased this risk beyond the effects of a lifestyle program, we undertook a parallel, 6-centre, randomised, double-blind, placebo-controlled phase 3 trial.

Participants were men, aged 50–74 years, waist circumference (WC) ≥ 95 cm, serum testosterone ≤ 14 nmol/L and with either impaired glucose tolerance (IGT) or newly diagnosed T2D assessed by Oral Glucose Tolerance Test (OGTT), (N=1007, 20% with T2D). All men were enrolled in a lifestyle program (WW) and randomised to receive intramuscular testosterone undecanoate 1000mg or placebo 3 monthly for 2 years.

At 2-years, in T (504) vs placebo (503) treated men: T2D in 12.4% (55/443) vs 21.1% (87/413) (p=0.0007), 2-h glucose 0.75 mmol/L lower than at baseline (p<0.0001), GTT normalised 51.9% vs 43.3% (P=0.012) and HbA1c was similar. The treatment effect was independent of baseline serum T and associated with a decrease in fat mass. Muscle mass and strength, bone density, erectile function, sexual desire and satisfaction increased significantly in T treated men. Lifestyle program engagement, quality of life and psychosocial function measures were similar in each group.

Harms (T vs placebo): SAEs: Cardiovascular (19 vs 19), BPH (8 vs 3), prostate cancer (4 vs 5), other cancers (10 vs 4), depression (1 vs 3), deaths (2 vs 2). Increased haematocrit ≥ 0.54 in 22% (106/491) vs. 1% (6/484), resulting in withdrawal of 25 men; and PSA in 23% (109/480) vs 19% (87/468).

Conclusion: Testosterone treatment for 2 years reduced T2D prevalence by 40% beyond the effects of a lifestyle program, an effect that is pharmacological, primarily mediated by favourable changes in body composition. Although without an increase in cardio-vascular risk, increases in haematocrit may be treatment limiting and longer-term durability and safety are uncertain.

Conclusion: Testosterone treatment for 2 years reduced T2D prevalence by 40% beyond the effects of a lifestyle program, an effect that is pharmacological, primarily mediated by favourable changes in body composition. Although without an increase in cardio-vascular risk, increases in haematocrit may be treatment limiting and longer-term durability and safety are uncertain.

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Diabetes Across the Lifecourse: A Translational Partnership to Improve Diabetes Outcomes Across Northern Australia

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In the context of the escalating epidemic of diabetes and related chronic conditions among Aboriginal and Torres Strait Islander people, it is vital that we reduce risk as early as possible in the life course. We have developed a partnership between researchers, health care providers, policy organisations and Aboriginal and Torres Strait Islander communities across Northern Australia, to address the issue of intergenerational diabetes in the high-risk population of these regions. The Diabetes across the Lifecourse: Northern Australian Partnership commenced in 2010 as the Northern Territory Diabetes in Pregnancy Partnership. Our work is now across the lifecourse, with a focus on youth and pregnancy; and we work across the Kimberley region of Western Australia, Northern Territory and Far North Queensland. The Partnership's Aboriginal and Torres Strait Islander Advisory group provides strategic advice concerning all aspects of the Partnership and provides advice and Indigenous knowledge on the best ways to ensure the research is conducted in a culturally appropriate way.

Our Partnership includes work with health service providers and Aboriginal communities to optimise antenatal care and to co-design and implement strategies to reduce diabetes-related risks before, during and after pregnancy, particularly among young women with type 2 diabetes. The PANDORA Study (Pregnancy And Neonatal Diabetes Outcomes in Remote Australia), a longitudinal birth cohort, sits within our Diabetes Lifecourse Partnership, and involves over 1100 women (of whom half are Aboriginal women), and their children. The third body of work in our Partnership involves work to co-design, implement and evaluate youth-friendly and culturally-appropriate models of care for Aboriginal and Torres Strait Islander children and youth with type 2 diabetes.

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Central administration of activin A in male mice suppresses food intake in response to fasting and ghrelin

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Normally, the brain maintains homeostatic control of body weight, successfully responding to changing energy needs. However, impaired appetite regulation, characterised by reduced appetite and body weight (cachexia-anorexia syndrome), occurs in many chronic disease states such as COPD, heart failure and cancer. Activin A (ActA), a member of the TGF-β family, is elevated in many chronic inflammatory conditions, including the aforementioned. Elevated ActA/B levels are also present, alongside cachexia, in our pancreatic cancer mouse model. Associations between ActA and cachexia symptoms have been observed, with heightened ActA levels correlating with weight loss, muscle degradation and mortality. As appetite regulation occurs in the brain and the activin receptor, ActRIIB, is located in brain regions associated with appetite control, we investigated whether ActA affects appetite or feeding-related behaviour. Mice were fasted overnight and food intake measured at multiple time points following ActA injection (varying doses). An ICV ActA dose of 0.15 ug and 0.5 ug (delivered into the lateral ventricle) suppressed refeeding within 1 hour. However this effect was not observed following intraperitoneal ActA injection. Furthermore, 0.15 ug ICV ActA also suppressed ghrelin-induced food intake, with the greatest effect observed at 1 hour post ActA injection. To assess sickness behaviour in response to ActA, we performed open field tests and behavioural barcoding analysis. No differences were observed in locomotor activity or in any of the 8 selected behaviours analysed, indicating ICV ActA does not produce malaise. Finally, ICV ActA was administered to assess c-fos immunoreactivity, a marker for neuronal activation, in brain regions involved in appetite control. Our findings reveal a role for ActA in appetite regulation, and pose the question of whether chronically high ActA levels in conditions such as cachexia may be responsible in part for facilitating appetite dysregulation.

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Glucocorticoid receptor isoform expression in PBMCs is different in states of glucocorticoid excess and deficiency and associated with alterations in downstream gene signalling

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Introduction: Glucocorticoids exert pleiotropic effects on all tissues, are essential for life, and are used therapeutically for a range of conditions. Exposure to excess glucocorticoid (as commonly seen with therapeutic dosing) is associated with a range of serious

adverse events and increased mortality. Glucocorticoids act via the glucocorticoid receptor (GR), a nuclear transcription factor, to exert their actions. A range of GR splice and translational isoforms have been identified, expression of which have been shown to affect glucocorticoid sensitivity. To date, these have been characterised *in vitro* and *in vivo* in a range of healthy and diseased tissues, here we report the first *in vivo* study of GR isoform expression in PBMCs of patients with varying states of glucocorticoid excess and deficiency.

Methods: PBMCs were isolated from whole blood of adult patient with normal HPA axis, glucocorticoid deficiency and glucocorticoid excess. Proteins from cytoplasmic and nuclear lysates were extracted. GR protein isoforms were measured via western blot and normalised to β-actin expression. PBMC gene expression was measured via RNA-Seq.

Results: GR protein isoforms detected in PBMCs of each group included GRα-A, GRα-C, GRα-D1-3, GR-A and GR-P. All GR isoforms were downregulated in the exogenous glucocorticoid group with some differences between other groups for individual isoforms. Isoform expression was downregulated across the day in women with AI. GR isoforms were correlated with expression of both shared, and unique genes, and gene expression profiles were markedly different in AI compared to the other groups.

Conclusion: The presence of multiple GR isoforms in PBMCs may regulate the sensitivity and specificity of the response to glucocorticoids under different conditions and disease states. This may be an important consideration when assessing glucocorticoid responsiveness in patients with endocrine disorders associated with altered HPA function or requiring exogenous glucocorticoid treatment.

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PCOS associated gene *TOX3* regulates steroidogenic cell differentiation

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Polycystic ovary syndrome (PCOS) is one of the most common causes of infertility females, affecting around 10% of women of reproductive age. Hyperandrogenism (elevated androgen levels), is one of the main diagnostical characteristics of this syndrome. Despite the high incidence of the disease, the etiology of PCOS is not completely understood, but appears to depend on both genetic and environmental causes. DNA variants in *TOX3* locus have been associated with polycystic ovarian syndrome (PCOS) in several populations, but the exact functional mechanism of *TOX3* in this disease or in the gonadal context is unclear. Unlike mammals, chicken gonads are steroidogenically active during early embryonic stages, making them an attractive model to study steroidogenic metabolic diseases. We identified *TOX3* as a novel transcription factor expressed in chicken embryonic gonads. *TOX3* mRNA and protein are expressed in Sertoli cells of the developing testis, colocalizing with AMH, SOX9 and DMRT1. In addition, *TOX3* expression is negatively regulated by estrogens *in vivo*, but not induced during masculinization induced by estrogen inhibition. *In vivo* DMRT1 knock-down in male gonads resulted in a down-regulation of TOX3 expression, whereas DMRT1 over-expression caused an increase in TOX3 expression. In addition, DMRT1 ChIP-seq confirmed the binding of DMRT1 in the promoter region of the TOX3 gene, suggesting that DMRT1 directly regulates TOX3 expression. TOX3 over-expression in male gonads resulted in a significant decline in *CYP17A1* positive steroidogenic Leydig cells. Taken together, this data suggests that DMRT1 regulation of *TOX3* regulates expansion of the steroidogenic, either directly, via cell lineage allocation, or indirectly via signalling from the supporting to steroidogenic cell populations. Mutations in *TOX3* could lead to unrestrained steroidogenesis in the gonad and elevated androgens. In the ovary, this could result in PCOS.

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Identification of mineralocorticoid receptor antagonist-sensitive biomarkers for personalised therapy in heart failure

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Background Heart failure (HF) is a major public health burden. Mineralocorticoid receptor antagonists (MRAs) reduce hospitalisation and improve survival in patients with HF with reduced ejection fraction (HFrEF) but the widespread use of MRAs is limited by adverse effects including hyperkalaemia. The use of biomarkers to guide therapy may enable earlier treatment or minimise side effects from unnecessary therapies. We have previously described mineralocorticoid receptor responsive genes in monocytes/macrophages and hypothesised that response to MRAs can be detected in this cell population. Therefore, we aimed to identify biomarkers for predicting response to MRAs to facilitate personalised treatment of HF.

Methods We performed microarray-based transcriptome profiling of monocytes obtained from six patients with HFrEF, before and after three months of MRA treatment. Multidimensional scaling (MDS) was employed to visualise sample similarity followed by differential expression analysis. Top differentially expressed genes (DEGs) implicated in cardiovascular pathophysiology were validated by RT-qPCR.

Results Three patients demonstrated improvement in left ventricular ejection fraction (LVEF) after 12 months of MRA treatment (Group 1), whereas the other three patients exhibited minimal functional response to MRAs (Group 2). The MDS plot revealed a distinct separation of males from females. Paired analysis of Group 1 samples (before vs after MRA treatment) identified 319 unique DEGs (estimated fold-change ≥ 2, q-value < 0.05); 1 upregulated, 318 downregulated). Group 2 had 1 unique DEG. RT-qPCR confirmed down-regulation of 12 of the top DEGs in Group 1. Additionally, cluster differentiation (CD) markers *CD14*, *CD163* and *CD68* were upregulated in Group 1 but downregulated in Group 2.

Conclusion Our results show MRA treatment can significantly alter the monocyte transcriptomic profile. Further research is needed to establish the utility of these DEGs as biomarkers for early prediction of MRA treatment-response in patients with HF, and to define sex-specific differences.

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Single-cell RNA analysis reveals heterogeneous sub-populations in neuroendocrine prostate cancer

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Background: Patients with neuroendocrine prostate cancer (NEPC) have aggressive tumours that result in poor patient outcomes. NEPC exhibits heterogeneity at the histological level, but the molecular drivers and therapeutic implications of this heterogeneity are poorly understood. Single-cell technology enables the resolution needed to unveil the complex heterogeneity of NEPC that bulk RNA analysis could not detect.

Objective: Characterise the molecular heterogeneity of NEPCs used in single cell RNA sequencing to detect common phenotypes across patients and identify new therapeutic targets.

Methods: We performed single-cell RNA sequencing on a novel cohort of eight patient-derived xenograft (PDX) models that recapitulate the pathological and clinical heterogeneity of NEPC. Downstream analysis of single-cell data was first completed on individual samples to identify distinct sub-populations of cells within each tumour. Then, integration analysis was performed to identify common and unique neuroendocrine populations across the different tumours.

Results: Our pipeline allowed the single-cell RNA profiling of 17,760 cells captured from the eight NEPCs. We found that each tumour had between 3 to 8 different sub-populations, where each sup-population displayed distinct biological properties such as epithelial-mesenchymal transition, quiescence and stemness. Data integration of all samples revealed 18 sub-populations of tumour cells across all patients, of which 10 populations were primarily unique to one patient. Gene set enrichment analyses revealed heterogeneous expression of most cancer hallmarks and oncogenic pathways across sub-populations, although some pathways were common to several neuroendocrine sub-populations such as P53 and KRAS.

Conclusions: We demonstrate that NEPCs display intra- and inter- tumour heterogeneity at the single-cell level, but the molecular complexity of these tumours may make it challenging to identify a single target. Therefore, future work should continue investigating the heterogeneity of NEPC, and whether combination therapy or precision medicine may be more effective approaches for treating NEPCs.

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A biallelic variant in *MRPL50* is associated with syndromic premature ovarian insufficiency in twin sisters

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Premature ovarian insufficiency (POI) is a common cause of infertility in young women. POI is associated with menstrual disturbance (primary or secondary amenorrhea) with elevated gonadotropins before the age of 40 and affects up to 1 in 100 women. This condition is highly heterogeneous with over 50 causative genes known so far, however, these explain only a minority of cases. Using whole exome sequencing, we identified an *MRPL50* homozygous missense variant (c.335T>A; p.Val112Asp) shared by twin sisters presenting with POI, sensorineural hearing loss, kidney and heart dysfunction. The identified variant is rare, affects a highly conserved residue and is predicted to be detrimental via *in silico* analyses. *MRPL50* encodes a component of the mitochondrial ribosomal large subunit. Using quantitative proteomics on patient fibroblasts, we demonstrated a loss of *MRPL50* protein and an associated destabilisation of the large subunit of the mitochondrial ribosome while the small subunit was preserved. The mitochondrial ribosome is responsible for the translation of subunits of the mitochondrial oxidative phosphorylation machinery, and we found patient fibroblasts have a mild but significant decrease in mitochondrial complex I. These data support a biochemical phenotype associated with *MRPL50* variants. To validate the association of the *MRPL50* variants with the clinical phenotype, we are using RNAi and CRISPR-Cas9 mediated technology to generate knockdown and knockout/knockin *Drosophila melanogaster* disease models for our *MRPL50* variant, which affects a well-conserved residue in flies. Preliminary data indicate that flies with *MRPL50* knockdown have disrupted ovarian development/function. We will continue to monitor *MRPL50* fly models for fertility, ovarian morphology and the development of

both germline and somatic cells in ovaries. In conclusion, we have shown that a *MRPL50* missense variant destabilises the mitochondrial ribosome and leads to syndromic POI in humans. Our findings highlight the role of mitochondria in the development and function of human ovaries.

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Biomechanical response of bone to load and mechanobiological mechanisms of load induced OA

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Available Soon

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How Muscles Influence Bone Structure

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Musculoskeletal health is dependent on the physiological relationship between muscle and bone. Bone is known to be highly sensitive to mechanical and load-bearing forces, including voluntary forces exerted by skeletal muscle. Therefore, it is not surprising that muscle activity is often correlated with bone strength across all stages of life. Bones that are subjected to dynamic, mechanical forces, such as those generated during resistance and high-impact exercises, have been associated with favourable geometric properties and include increased cortical thickness and periosteal diameter. In contrast, a lack of muscle activity or function severely impairs skeletal development and drives the loss of bone during disease and immobilisation.

The direct influence of muscle activity on the adjacent bone remains incompletely understood, as it is inherently difficult to separate the influence of muscles from the local and systemic effects of load-bearing forces and physical activity. However, some studies have been able to manipulate muscles independently of exercise and have since provided interesting insight on how muscle size and muscle contractions refine bone structure. Muscle size and function have been shown to play crucial roles in maintaining the characteristic shape of the tibia in rodents and humans, while also contributing to the formation of entheses during skeletal development. Additionally, our recent work has also shown that rapid muscle growth in sedentary, adult mice, initiated cortical modelling and altered bone structure. Further studies are still required to fully understand the physiological relevance of rapid changes in muscle size on bone during development, adulthood and ageing.

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Systematic Review: Morbidity and all-cause mortality of current osteoporosis treatments.

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Background: Osteoporosis is an incurable, progressive, chronic disease that requires long-term treatment. Recent studies have brought the long-term safety of the pharmacologic treatment of osteoporosis into question.

Aim: To investigate and clarify the morbidity (serious adverse events) and all-cause mortality of commonly used anti-osteoporosis drugs.

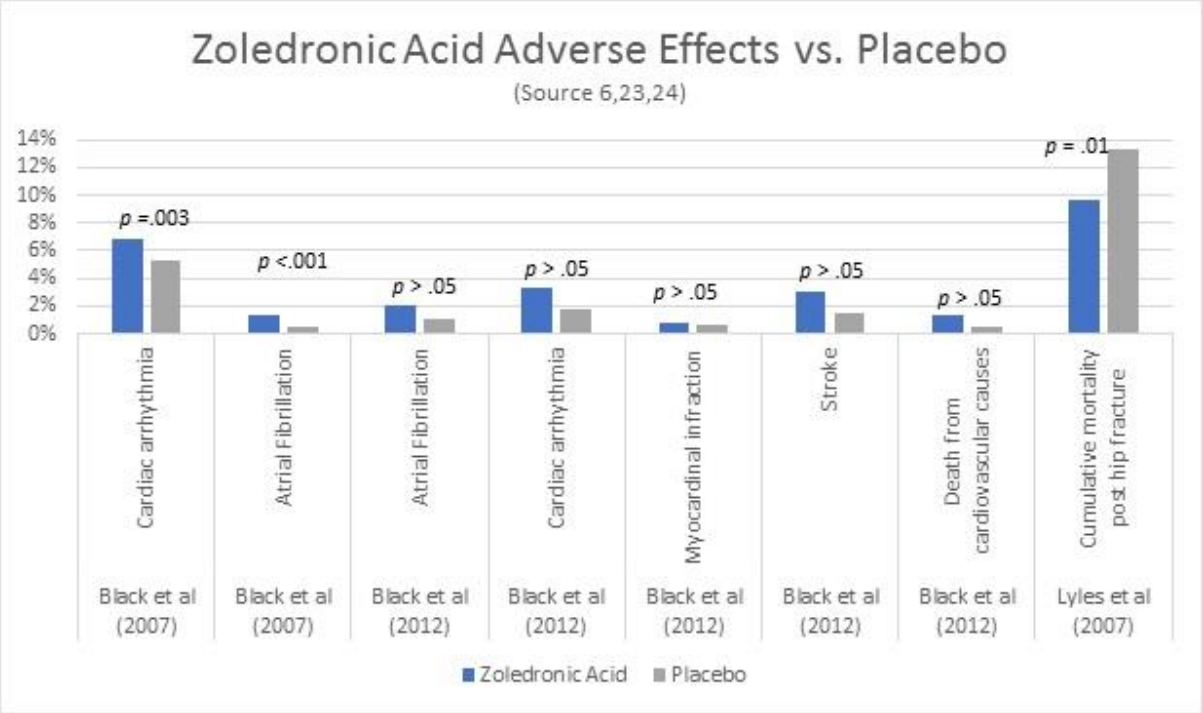
Methods: PubMed and Cochrane electronic databases for searched for clinical trials that enrolled osteoporotic men and postmenopausal women. Randomised controlled trials, post hoc analysis of randomised controlled trials and cohort studies published after 1990 were selected. These included placebo-controlled, active-controlled and head-to-head studies.

Results:

Raloxifene (selective serotonin receptor modulator) increases the risk of thromboembolic events 2-3-fold compared to placebo but reduces the risk breast cancer by more than 75%. In patients with cardiovascular risk, the reduction in arterial adverse events outweighs the increased risk of venous thromboembolic adverse events to significantly reduce morbidity. Bisphosphonates and Denosumab have a serious adverse event profile similar to that of placebo for up to 10 years of continuous use. The risk of osteonecrosis of the jaw and atypical femoral fracture with antiresorptive therapy is low but numerically increases with cumulative dose and duration. However, the benefits of denosumab are rapidly reversible after cessation increasing fracture risk. Bisphosphonates may increase arrhythmia risk (3% vs 1.8%) but reduces cardiovascular mortality by up to 30% at 3 years. Romosozumab is inferior to alendronate with regards to cardiovascular and cerebrovascular risk.

Conclusion: This review moderates concerns about the safety of bisphosphonates and additionally demonstrates that zoledronic acid and alendronate appear to have a cardiovascular mortality and major morbidity benefit. Commonly used anti-osteoporosis drugs are safe and well tolerated with a favourable serious adverse event profile. Raloxifene has an overall cardiovascular benefit. Bisphosphonates may increase arrhythmia but have a mortality benefit. Denosumab benefits are precipitously reversible on

discontinuation.



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Accelerated diffuse osteosclerosis, high bone mass phenotype and hungry bone syndrome in a man with adenocarcinoma of unknown primary

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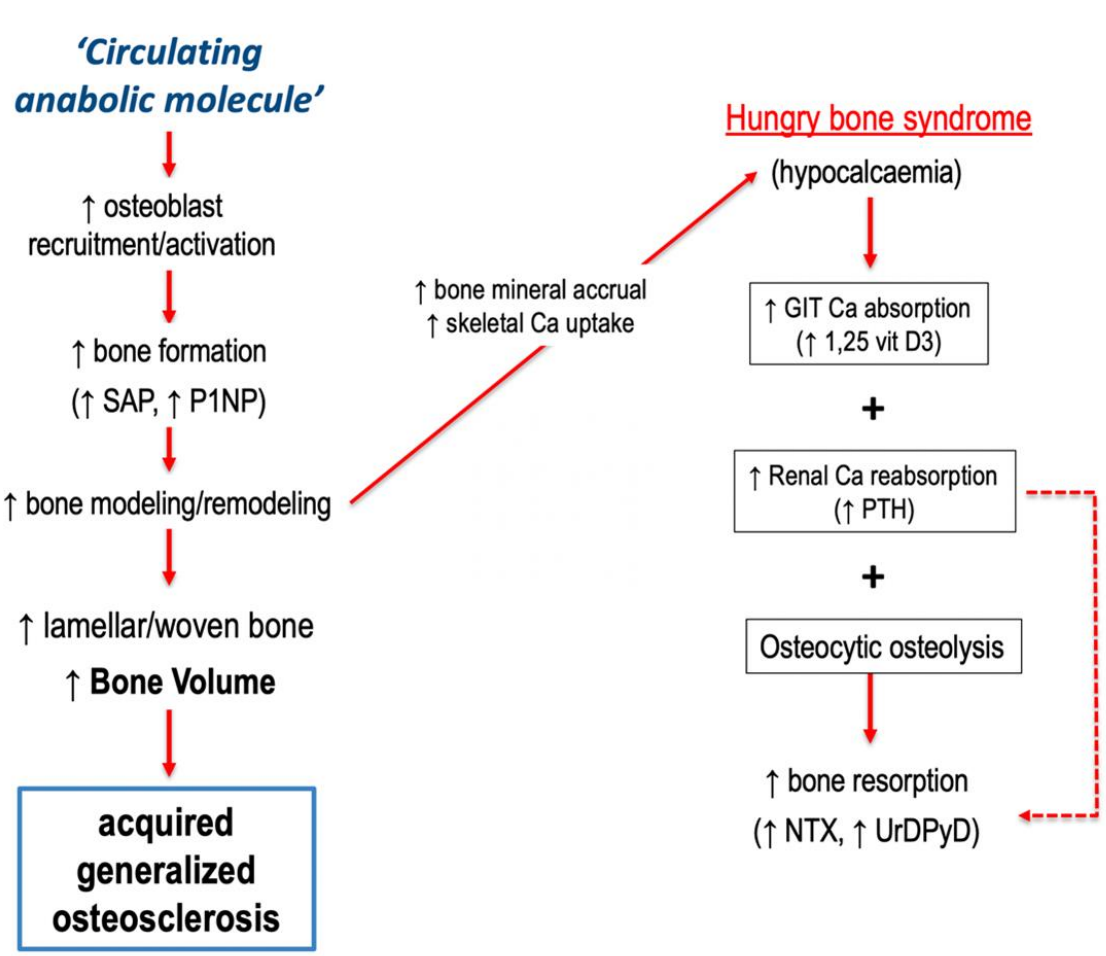
A 71-year-old man was referred for evaluation of incidental generalised osteosclerosis. He was found to have high bone mass (HBM) with an elevated lumbar spine bone mineral density (BMD) measured by dual-energy X-ray absorptiometry (DXA) (1.79 g/cm^2) (Z-score +4.6)), a dramatic increase of 62.7% compared to 18-months prior. Left femoral neck BMD also increased by 17.8% from 0.83 g/cm2 to 1.01 g/cm^2. Lumbar spine BMD measured by quantitative computed tomography (QCT) was 416.4 mg/cm^3 (Z-score +10.9). Biochemical markers of bone formation and resorption were markedly elevated. Rapid skeletal calcium uptake resulted in profound serum hypocalcaemia (1.88 mmol/L) and hypocalciuria (<0.2 mmol/day) suggestive of hungry bone syndrome (HBS). Screening investigations for acquired causes of osteosclerosis and whole-genome sequencing for common pathogenic sclerostin gene variants were negative. CT pan-scan demonstrated small bilateral pleural effusions. Positron emission tomography (PET) with 2-deoxy-2-[fluorine-18] fluoro-D-glucose (^{18}F-FDG) confirmed diffuse osteosclerosis with no focal abnormal FDG uptake in the skeleton or elsewhere to suggest an underlying primary malignancy or metastatic disease. Bone biopsy showed markedly sclerotic woven and lamellar bone, wide osteoid seams and osteocytic osteolysis. The marrow space was devoid of typical bone cells and adipocytes and instead filled by fibro-myxoid stroma, infiltrated by small clusters of adenocarcinoma tumour cells. Bone histomorphometry demonstrated elevated trabecular bone volume. Adenocarcinoma cells were also discovered in pleural fluid cytology and skin verruca histology. He deteriorated over the following 6-months despite chemo/immunotherapy whilst lumbar spine QCT BMD increased further to 548.9 mg/cm^3 (Z-score +13.2). He passed away 9-months post-diagnosis.

The bone disorder in this case is unique and raises the possibility of a novel anabolic factor(s) secreted by an adenocarcinoma of unknown primary resulting in dramatic increases in BMD, HBM and radiological osteosclerosis. The differential diagnosis and potential mechanisms responsible for HBM will be discussed in the presentation.



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Comparison of Fracture Rates and Economic Outcomes Between Women with Osteoporosis Receiving Risedronate Enteric-Coated and Alendronate

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Objective: Risedronate Enteric-Coated (EC) offers a convenient dosing option eliminating the need for fasting, while providing a higher bioavailability of the medication when compared to immediate-release risedronate (*Risedronate EC SmPC)*. However, the benefit of this dosing to reduce risk of fracture compared to other oral bisphosphonates such as alendronate remains unknown. Thus, the aim of this study was to compare the risk of fractures and economic outcomes between women with osteoporosis receiving risedronate EC vs. alendronate immediate-release.

Methods: Women with osteoporosis were identified from a US claims database (2009-2019) and observed for ≥2 years following the date of their first observed dispensing for an oral bisphosphonate (index date). Women were classified into the risedronate EC or alendronate cohort based on the treatment-initiated index date. The cohorts were matched 1:1 based on demographic and clinical characteristics evaluated during a six-month period prior to the index date. Incidence rates (IRs) of fractures and healthcare resource utilization per 1,000 patient-years were compared between the two cohorts using IR ratios (IRRs). Alpha level set as *P<0.05*.

Results: 1,807 patients were selected in each cohort (median age: 60.0 years; average observation period [years]: risedronate EC: 4.3, alendronate: 4.6). The IR of fractures was significantly lower in the risedronate EC vs the alendronate cohort for any fracture site (IRR: 0.81, 29%, reduction, p<0.05) and spine fractures (IRR: 0.69, 31%, reduction *p<0.05*) (table-1). Compared to the alendronate cohort, the risedronate EC cohort incurred fewer hospitalizations (IR, EC: 112.03; alendronate: 134.69; IRR: 0.85, *p<0.05*) translating into numerically lower hospitalization costs (average per-patient-per-year; EC: $3,605; alendronate: $4,572, *p=0.07*).

Conclusion: Findings indicate that women treated with risedronate EC have a lower incidence of fractures compared to those treated with alendronate, consistent with the hypothesis that the enteric-coated formulation of risedronate (35mg once weekly) improves medication absorption, enabling greater effectiveness.

Table 1: Fracture incidence rate (IR) of Risedronate EC and immediate-release Alendronate

	IR Risedronate EC (N=1,807)	IR Alendronate (N=1,807)	IRR (95% CI)
Any site	33.97	42.53	0.81 (0.66 - 0.98) *
Hip	9.21	9.61	0.99 (0.65 - 1.51)
Pelvis	2.07	3.12	0.68 (0.35 - 1.33)
Spine	10.76	15.86	0.69 (0.49 - 0.97) *
Wrist/arm	14.52	15.86	0.91 (0.70 - 1.20)

*$p \leq 0.05$

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Lithium use and bone mineral density

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Aim: Psychiatric disorders and most medications used to treat them have been previously shown to be independently associated with skeletal deficits. In contrast, there is increasing evidence suggesting lithium may possess skeletal protective properties. Thus, we aimed to investigate the association between lithium use and bone mineral density (BMD) in a sample of women with bipolar disorder.

Method: Women with a history of bipolar disorder (n=117) were recruited from the Barwon Statistical Division, south-eastern Australia. Bipolar disorder was confirmed using a semi-structured clinical interview (SCID-I/NP). BMD (g/cm^2) was measured at the spine, hip and total body using dual-energy X-ray absorptiometry (Lunar). Weight and height were measured and information on medication use and lifestyle variables were obtained via questionnaire. Socioeconomic status (SES) was determined. Linear regression models were used to test associations between lithium use and BMD, after adjusting for age, weight and medication known to affect bone.

Results: Thirty five (29.9%) women reported current lithium use. Lithium users and non-users differed in regards to SES (p=0.03) and BMD at the hip (p=0.03); otherwise the groups were similar in age, weight, height, smoking status, activity levels, alcohol and calcium intake and BMD at the spine and total body. After adjustments, mean BMD among lithium users was 5.0% greater at the spine [1.275 (95% CI 1.229-1.321) vs 1.214 (95% CI 1.183-1.244) g/cm^2, p=0.03], 4.2% greater at the hip [0.979 (95%CI 0.942-1.016) vs 0.938 (95%CI 0.910-0.966) g/cm^2, p=0.03] and 2.2% greater at the total body [1.176 (95% CI 1.148-1.205) vs 1.150 (95%CI 1.129-1.171) g/cm^2, p=0.08] compared to non-users. Smoking, physical activity, alcohol and calcium intake, SES and other psychotropic medications did not contribute to the models.

Conclusion: These data suggest lithium use is associated with greater BMD in women with bipolar disorder. Replication and research into underlying mechanisms are warranted.

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Estimating relative fracture reduction of romosozumab versus teriparatide for postmenopausal osteoporosis using bone mineral density outcomes

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INTRODUCTION: The aim of this study was to use direct bone mineral density (BMD) outcomes for romosozumab versus teriparatide to estimate relative risks (RRs) of fracture in postmenopausal women with osteoporosis. The approach is justified by the significant linear relationship between percentage total hip BMD change from baseline and RRs of hip and vertebral fracture on the log scale shown by a meta-regression conducted by the Foundation for the National Institutes of Health (FNIH).

METHODS: The STRUCTURE trial provides a 3.4% difference in total hip BMD change from baseline at 12 months for romosozumab versus teriparatide in patients previously treated with bisphosphonates. This value was translated into RRs of hip, vertebral, and nonvertebral fracture using slopes from the FNIH meta-regression. Uncertainty around resulting estimates was derived by error propagation. As a sensitivity analysis, BMD efficacy for romosozumab versus teriparatide was taken from a phase II trial (2.8% difference in total hip BMD change from baseline) to explore outcomes for a treatment-naïve population.

RESULTS: In the base case, RRs of fracture (with 95% confidence intervals) for romosozumab versus teriparatide were 0.75 (0.60 to 0.95), 0.53 (0.37 to 0.76), and 0.90 (0.78 to 1.03) for hip, vertebral, and nonvertebral fracture, respectively. The sensitivity analysis using phase II trial data yielded RRs of 0.79 (0.64 to 0.97), 0.59 (0.43 to 0.82), and 0.91 (0.81 to 1.03) for hip, vertebral, and nonvertebral fracture. Validation of the approach using romosozumab trials reporting both BMD and fracture outcomes at 12 months (FRAME, ARCH) showed consistency between BMD-predicted and observed RRs of fracture.

CONCLUSION: Results indicate that the BMD benefit of romosozumab versus teriparatide translates into clinically meaningful fracture reductions for bisphosphonate pre-treated and treatment naïve patients.

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Targeting Notch2 signalling to overcome methotrexate chemotherapy-induced bone and bone vasculature damage in rats

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Childhood methotrexate (MTX) chemotherapy often causes skeletal complications such as osteopenia and bone vasculature dysfunction. However, the precise molecular mechanism of damage is not completely understood and there is a lack of appropriate treatment. Notch signalling is one of the key signalling pathways known to regulate cell fate decision, proliferation and differentiation. Knowing the fact that bone vasculature plays crucial roles in bone turnover and bone homeostasis, whether and how Notch signalling pathway deregulation plays a role in cancer chemotherapy-induced bone damage and its correlation with vasculature disfunction is unknown.

Time course analyses of long bones from rats receiving intensive MTX treatment (5 consecutive daily doses of 0.75 mg/kg, which mimics childhood acute lymphoblastic leukemia treatment) found decreased trabecular bone volume, vasculature dilation and regression at day 9 following the first MTX dose. For exploring potential mechanisms, PCR array screening of the gene expression for 92 key factors regulating bone and vasculature homeostasis revealed increased expression of *NOTCH2* after MTX treatment. Consistently, increased activity of Notch2 was confirmed by increased Notch2 intracellular domain protein level and by upregulation of Notch target genes in metaphysis at days 6 and 9 following the first MTX administration. To confirm the roles of Notch2 signalling in MTX bone and vasculature damage, a neutralising anti-Notch2 antibody or a control IgG was administrated during MTX treatment. Micro-CT analyses demonstrated that trabecular bone volume was preserved by the MTX+anti-Notch2 antibody treatment when compared to the MTX+Control IgG. Also, there was a significant increase in the volume of vasculature canals in the MTX+Control IgG treatment group compared to the control. However, blockade of Notch2+MTX treatment attenuated this increase. Our results suggest that Notch2 signalling plays an important role in mediating MTX treatment-induced bone damage/vasculature dysfunction and targeting Notch2 could be a potential treatment against MTX skeletal side effects.

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Regulation of medullary bone in Japanese quail requires photoperiod-induced central actions of thyroid hormone.

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Seasonal reproduction enables animals outside tropical regions to rear offspring in a favourable environment. Increasing day length triggers a hypothalamic relay involving thyrotropin, type-2 deiodinase and thyroid hormone, which activates the hypothalamic-pituitary-gonadal axis to induce reproductive competence. Photoperiod regulates calcium metabolism and egg-laying in Japanese quails (*Coturnix japonica)*. We hypothesised that activity of this relay would have major consequences for the skeleton. Quails were housed in long (20h light/4h dark) or short (6h light/18h dark) day conditions for up to 12 weeks. Skeletal consequences were determined by X-ray microradiography, micro-CT, electron microscopy, histomorphometry and biomechanical testing (n=10/sex/group). Both ovary and testis weights increased >10-fold (P<0.001, ANOVA) after exposure to long days compared to short days. Long day females displayed massive increases in bone mineral content (P<0.001, Kolmogorov-Smirnov; KS), and bone strength and stiffness (P<0.001, ANOVA), due to medullary bone formation. In contrast, medullary bone was absent in short day females and never seen in males. Medullary bone was highly vascular and dynamic, with osteoclast resorption pits and mineral apposition fronts covering almost the entire bone surface. Reversal of photoperiod resulted in (i) rapid ovarian regression and loss of medullary bone in females previously exposed to long days, and (ii) rapidly increased ovarian size and medullary bone formation in females previously exposed to short days. Overall, the skeleton is exquisitely sensitive to photoperiod during avian seasonal reproduction, and central actions of thyroid hormone are essential for

medullary bone formation. Furthermore, to investigate the dynamics of calcium mobilisation and eggshell formation, long day females were examined every 4 hours during one 24h reproductive cycle. Bone mineral content was highest after ovulation, and decreased rapidly during shell calcification (P<0.001, KS). Elucidation of the cellular mechanisms involved may identify novel pathways coupling rapid formation/resorption of medullary bone; an important model for osteoporosis.

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Drug targeting of Tyrosine kinase receptor c-ros-oncogene 1 (C-ROS-1) to treat pre-fusion of coronal sutures in a pre-clinical model of craniosynostosis

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Craniosynostosis is the premature bone fusion of the skull plates resulting from deregulated proliferation and differentiation of cranial suture osteogenic stem cells into bone. Children with craniosynostosis exhibit increased intracranial pressure leading to neurological deficits. Current treatment options rely on major invasive cranial surgery. Saethre-Chotzen syndrome (SCS), defined by a loss-of-function mutation in the *TWIST-1* gene, is one of the most prevalent craniosynostosis (incidence 1/25,000 births[1]). One gene known to promote osteogenic differentiation of *TWIST-1* haploinsufficient cranial cells is the transmembrane tyrosine kinase receptor C-ROS-1 [2]. *In vitro* studies have demonstrated a reduced osteogenic potential of these cells following inhibition of C-ROS-1 activity with the tyrosine kinase inhibitor, Crizotinib[2]. The present study assessed the efficacy of Crizotinib to halt premature coronal suture fusion in a pre-clinical mouse model of SCS. Crizotinib at different concentrations was administered over the calvaria of Twist-1$^{del/+}$ heterozygous mice at postnatal day 8 (P8), prior to coronal suture fusion, using either a non-resorbable collagen sponge (quick drug release) or a resorbable sodium carboxymethylcellulose (CMC) microdisc (slow sustained release). Coronal suture fusion rates and bone parameters were determined by µCT and histomorphometric analysis of calvaria at P20 and P25 (post coronal suture fusion). Results demonstrated a dose dependent increase in the efficacy of Crizotinib to maintain coronal suture patency by P20, with no adverse affects to brain tissue or blood cell parameters. Moreover, Crizotinib delivered on CMC discs resulted in a greater efficacy to reduce bone formation at the coronal suture sites by P20 compared to its delivery on sponges. Whilst a single application of Crizotinib at P8 was effective at halting suture fusion, this effect was reversible and the bone inhibitory effects diminished by P25. Our findings lay the foundation for the development of a pharmacological targeted approach to treat craniosynostosis.

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Integrating phosphoproteomics and functional genomics to identify novel insulin signalling regulators in bone

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Insulin signalling in bone plays a critical role in development and the regulation of energy metabolism. However, a systems biology analysis to map *in vivo* signalling has yet to be performed. Furthermore, whether signalling is rewired during ageing and insulin-resistance is unknown. We present the first mouse bone phosphoproteome of 8- and 73-week-old mice following acute *in vivo* insulin stimulation and identified >16,000 phosphorylation sites of which >4,600 are novel. We observed hundreds of phosphorylation sites differentially regulated between young and old bone revealing dramatic rewiring and defects in insulin signalling. Machine learning coupled to evolutionary conservation analysis and integration with human GWAS enabled us to prioritise novel kinase substrates highly likely to play important roles in bone function. We next developed a semi-high throughput CRISPR/Cas9 loss-of-function screen in zebrafish to interrogate these novel phosphorylation events and the regulation of bone formation. We present ongoing results from this functional screen and we hope our functional analysis of the phosphoproteome will further enhance our understanding of the signalling mechanisms controlling bone biology and whole-body energy metabolism.

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Deletion of leptin receptor further delays cortical bone consolidation in female mice with activated STAT3 signalling

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Bone strength is partially determined during corticalisation, a two-step process comprising coalescence of peripheral trabecular bone and its progressive mineralisation. Hyperactivation of STAT3 phosphorylation in osteocytes, by targeted deletion of suppressor of cytokine signalling 3 (Socs3), delays corticalisation leading to high cortical porosity, low mineralisation and low bone strength. Since multiple bone-active cytokines activate STAT3 including leptin, IL-6 family cytokines, and G-CSF, we evaluated whether blocking leptin signalling in osteocytes of Dmp1cre^{Tg}.Socs3$^{fl/fl}$ mice would rectify their cortical bone deficit.

We generated mice deficient in both Socs3 and Leptin Receptor (LepR), using Dmp1cre^{Tg}. Dmp1cre^{Tg}.Socs3$^{fl/fl}$.LepR$^{fl/fl}$ mice (DSL) were compared to cousin Dmp1cre^{Tg}.Socs3$^{fl/fl}$ (DS) controls by micro-computed tomography. We also generated Dmp1cre^{Tg}.LepR$^{fl/fl}$ mice to assess the effect of leptin signalling at physiological STAT3 levels, using Dmp1cre^{Tg} as controls; these mice showed no detectable bone phenotype.

Between 6 and 12 weeks of age, although corticalisation was delayed, male and female DS control mice showed some maturation of the cortex, including a 64% reduction in cortical porosity and a 200% increase in high-density bone. Six-week-old DSL mice showed the same phenotype as DS control mice. At 12-weeks of age, male DSL and DS mice showed the same pattern of skeletal maturation, however, female DSL mice showed no reduction in cortical porosity since 6 weeks, and a 32% greater proportion of low-density bone mass than age-matched DS controls. Between 6 to 12 weeks, female DSL mice accrued half the amount of medium- and high-density bone than that accrued during the same period in DS mice. This indicates that absence of LepR in mice with STAT3 hyperactivation further delays bone corticalisation.

This identifies a new role for leptin signalling in bone. Although LepR within osteocytes had no irreplaceable physiological role in cortical bone development, in the context of STAT3 hyperactivation, leptin receptor signalling promotes cortical bone consolidation.

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Lymphocytes are not required for the development of neurogenic heterotopic ossification after spinal cord injury

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Neurogenic heterotopic ossifications (NHO) are incapacitating complications of traumatic brain and spinal cord injuries (SCI) which manifest as abnormal heterotopic bones in periarticular muscles. NHOs are debilitating, causing pain, joint ankylosis, as well as vascular and nerve compression. The mechanisms leading to NHO are unknown and the only effective treatment remains surgical resection. To elucidate NHO pathophysiology we developed the first model of NHO following SCI in genetically unmodified mice. Using this model, we established that the innate immune system plays multiple roles in NHO pathogenesis. We demonstrated that macrophage / monocyte release of oncostatin M and subsequent JAK/STAT3 signalling in injured muscles promoted NHO development and treatment with the JAK1/2 tyrosine kinase inhibitor ruxolitinib significantly attenuated NHO[1,2,3]. Both clinical and experimental studies have previously established that SCI results in depressed adaptive immune responses and reduced lymphocyte frequency, however, the contribution of adaptive immune cells to NHO pathogenesis remains largely unexplored. We established that T cell subsets are present in muscles after SCI (at T12-T13 vertebrae level), or muscle damage, but there was no significant changes in T cell frequency between surgical groups when compared to naïve mice. In contrast, B cells were significantly increased in muscles developing NHO. We also noted a reduction in B cell frequency in blood and splenic B cell frequency was reduced after SCI compared to muscle injury alone. Interestingly, mice that underwent splenectomy prior to SCI and muscle damage developed NHO volumes similar to Sham operated mice. Finally, to determine if adaptive immune cells play a direct role in NHO pathogenesis, *Rag1*$^{-/-}$ mice (defective for mature T and B lymphocytes), developed NHO with volumes similar to wild type mice. Overall, these findings suggest that functional T and B cells have minimal influence or at most make redundant contributions to NHO development following SCI.

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Microfluidics for Fertility

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Microfluidics offers several advantages over their traditional macro-scale counterparts by extending the possibility of biomedical research based on the idea of miniaturization. These platforms provide opportunities to manipulate cells and biological processes at the single-cell level and develop nature-inspired technologies for biomedical applications. For example, in the context of fertility, microfluidics can match the microgeometry of the female reproductive tract to present opportunities for biomimicry-based selection of sperm that reflect the in vivo process. In this talk, I will provide an overview of our work in developing microfluidic technologies for understanding human reproduction, and sperm analysis and selection. These platforms present several promising avenues to address our large-scale infertility challenges.

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The impacts of *Chlamydia* infection and vaccination on the male reproductive tract

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Despite being the most common bacterial sexually transmitted infection worldwide, with 127 million new infections per year, a Chlamydia vaccine remains elusive. Vaccination remains the most highly recommended method for eliminating chlamydial transmission and subsequent pathology, the most severe of which is infertility. Research has predominantly focused on females even though a similar incidence of infection occurs in both sexes, resulting in potential vaccine candidates being trialled only in females. Male infections are often underappreciated as the effects on male fertility are still being defined. Some data suggests that Chlamydia causes sperm DNA fragmentation, which is an important clinical indicator of fertility.

We developed a male mouse model of prophylactic chlamydial vaccination. Male C57BL/6 mice were vaccinated intra-nasally with major outer membrane protein combined with Iscomatrix adjuvant (MOMP/IMX) or with IMX only, then challenged via the intra-penile route with Chlamydia muridarum, or PBS. At 1-, 2-, and 3-months post challenge the IMX adjuvant-only group had poor sperm quality demonstrated by lowered motility and oocyte-binding, abnormal morphology, and increased DNA damage. However, the MOMP/IMX vaccination group had comparable sperm quality to the non-infected control mice. Vaccination protected against infection-induced impairment of sperm motility, morphology, oocyte-binding, and DNA damage. Testicular and epididymal chlamydial burden were also reduced by vaccination. We've shown that vaccine-mediated protection is induced by multifunctional CD4+ and CD8+ T cell involvement combined with local and system IgG and IgA production in the reproductive tract, which protect normal testicular function.

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Developing Novel Devices and Treatments to Improve Pregnancy Outcomes

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Introduction

Pregnancy and birth are among the most dangerous days in your life. Stillbirth tragically ends 3 million pregnancies globally every year whilst fetal asphyxia inflicted by labour is a leading cause of neonatal seizures, cerebral palsy and death. Pregnancy can also be detrimental to the mother and multisystem organ injury can result from preeclampsia, with delivery, often at preterm gestations, being the only treatment.

Unfortunately, current measures of fetal wellbeing during pregnancy and direct measures of fetal distress during labour are intermittent and often miss the critical point when a life-saving birth could be performed. Excitingly we are developing two devices to continuously measure markers of fetal distress in pregnancy and directly measure markers of fetal distress during labour. Furthermore, we have identified a possible medical treatment for preeclampsia, metformin.

Methods

With a team of electronic, material and chemical engineers and physicists we are using novel flexible electronics and artificial intelligence to develop a device to detect markers of fetal well-being non-invasively in pregnancy. Utilising cutting edge fibre optic and sensor technology we are developing a device to accurately measure direct markers of fetal distress during labour. We identified metformin as a possible treatment for preeclampsia in laboratory assays and have performed a randomised control trial evaluating its potential as a treatment for preterm preeclampsia.

Results

We recruited 150 pregnant women and discovered that 6 sensors positioned around the maternal umbilicus obtain a reliable fetal ECG. On implementing artificial intelligence, we were able to reliably extract the fetal ECG 97% of the time compared to traditional algorithms at 90% ($p<0.05$). We have developed a wearable sensor and wireless, portable hardware that is smaller and lighter than a smart phone that could be worn in pregnancy to continuously determine fetal well-being.

We have optimized the physics and chemistry of our sensor that could be used in labour and demonstrated it accurately detects a marker of fetal distress in buffers. We have promising data showing it accurately detects markers of fetal distress in biological samples.

We have shown metformin reduces antiangiogenic markers of preeclampsia in laboratory assays. We have performed a randomized clinical trial enrolling 180 women with preterm preeclampsia and excitingly demonstrated metformin prolonged pregnancy by 7 days ($p = 0.056$).

Conclusion

We are developing technology to continuously assess fetal well-being in pregnancy and labour and have discovered a possible treatment for preeclampsia. These devices and treatment have the potential to detect fetal distress and subsequently reduce stillbirth and hypoxic complications of labour and preeclampsia.

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Examining the impacts of toxins on female fertility and health of future generations

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The female germline is exceedingly sensitive to the exposure to toxicants, including those found in the environment and those used to treat diseases such as cancer. Oocytes are one of the longest living cells in the mammalian female body, and may have prolonged exposure to exogenous and endogenous toxicants that can cause DNA damage and instability. For the females of many species, perturbations caused by toxicants result not only in reproductive disorders, such as infertility and premature ovarian failure, but also in non-reproductive metabolic and health-related diseases. Of further concern is that these adverse effects can also be passed onto offspring via genetic and non-genetic (epigenetic) inherited damage sustained in the oocytes. Using a combination of genetic mouse models, chemotherapies, radiation and environmentally relevant concentrations of pesticides my work aims to i) identify mechanism's involved in preserving fertility, ii) understand how genome integrity is maintained in oocytes and iii) determine if mechanisms used to preserve fertility are safe with respect to genome integrity and future generation's health. Understanding the effects of life saving chemotherapies and drugs in addition to pervasive environmental toxicants on the ovary, oocytes, and female fertility is vital, as perturbations can result in reproductive disorders and systemic diseases in current and future generations.

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Lower Psoas Muscle Area is associated with increased mortality after endovascular aneurysm repair in older adults

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Association between the lowest tertile of PMA for either sex with 5-year mortality with 5-year mortality after EVAR procedure

	HR (95% CI)	P-value
Unadjusted	2.31 (1.02, 5.24)	0.045
+ age	2.01 (0.87, 4.64)	0.101
+ weight, age and sex	2.50 (1.03, 6.07)	0.043
+ BMI, age and sex	2.76 (1.08, 7.03)	0.034
+ obesity (BMI = 30)	2.94 (1.17, 7.39)	0.022
+ modified Frailty Index	2.30 (0.99, 5.34)	0.054

Introduction: Psoas muscle area (PMA) is an easily measured surrogate marker of sarcopenia. However, evidence is conflicted as to whether PMA is associated with poorer post-operative outcomes. This study aimed to further explore the relationship between PMA and outcomes post asymptomatic infrarenal endovascular aortic repair of aneurysm (EVAR).

Methods: A retrospective observational study reviewed people aged over 65 years undergoing EVAR from 1/1/2013 to 31/12/2017. PMA was measured at mid L4 vertebral body on preoperative computer tomography scan. Primary outcome was survival in days from operation. Secondary outcomes were 30-day readmissions related to EVAR, post-operative complications and length of stay measured in days.

Results: Ninety-seven patients (mean age 77.5 years, 78% male) were assessed. Patients in the lowest PMA tertile had an increased unadjusted 5-year mortality (HR 2.31, 95%CI 1.02-5.24, p=0.045). Adjustment for BMI showed also an increased 5-year mortality (HR 2.91, 95%CI 1.16-7.34, p=0.023). Adjustment for age, sex and BMI also showed that patients in the lowest PMA tertile had an increased 5-year mortality (HR 2.76, 95%CI 1.08, 7.03; P=0.034). Adjustment for frailty, in the form of modified frailty index, showed only a minimal attenuation of the association (HR 2.30, 95%CI 0.99, 5.34; p=0.054).

After adjusting for BMI patients with the lowest tertiles of PMA for either sex had increased total LOS (β 0.33, 95%CI 0.04, 0.63; P=0.028). Risk of death was also increased (OR 3.53, 95%CI 1.21, 10.27: P=0.021). 30-day readmissions, complications and discharge destination were not influenced by PMA.

Conclusion: The lowest tertile of PMA, as a surrogate marker for sarcopenia, was associated with increased mortality in patients undergoing EVAR. Adjustment for frailty showed a minimal attenuation of this association Although sarcopenia is a complex

phenomenon and the literature surrounding it is still evolving, PMA may form one component of risk assessment in patients undergoing EVAR.

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Markers of higher bone turnover are associated with all-cause and cardiovascular disease mortality risk in older men.

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Background:
Osteocalcin in its undercarboxylated form (ucOC) may influence diabetes risk, however its relationship with all-cause and cardiovascular disease mortality is unclear. Whether other bone turnover markers (BTMs) are associated with mortality risk differently from ucOC also remains uncertain.

Objective:
To determine associations of serum ucOC with all-cause and cause-specific mortality, and compare these with the corresponding associations of serum total osteocalcin (TOC), procollagen type 1 N-propeptide (P1NP) and collagen type 1 C-terminal cross-linked telopeptide (CTX).

Design, setting and participants:
Prospective cohort study of 3,871 community-dwelling men, aged 77.0±3.6 years at baseline, followed for a median of 12.3 years.

Methods:
Serum ucOC, TOC, P1NP and CTX were assayed. Incidence of all deaths, and deaths due to cardiovascular disease (CVD) or cancer, were ascertained by data linkage. Cox regression analyses were adjusted for cardiovascular risk factors.

Results:
Men in the highest quartile of ucOC had higher all-cause and CVD mortality compared to those in Q1 (Q4 vs Q1: hazard ratio [HR]=1.17, 95% confidence interval [CI]=1.02-1.35, p=0.029; HR=1.25, CI=1.01-1.54, p=0.037, respectively). Similar results were seen for TOC (HR=1.20, CI=1.04-1.39, p=0.016; HR=1.27, CI=1.03-1.58, p=0.029, respectively) and CTX (HR=1.24, CI=1.07-1.44, p=0.004; HR=1.27, CI=1.03-1.57, p=0.028). P1NP was associated with all-cause (HR=1.20, CI=1.04-1.38, p=0.013) but not CVD mortality. Only P1NP and CTX were associated with cancer mortality (HR=1.32, CI=1.01-1.72, p=0.042; HR=1.37, CI=1.04-1.80, p=0.023 respectively).

Conclusions:
Higher bone turnover, assessed by ucOC and other BTMs in older men, is a biomarker for all-cause and cause-specific mortality risk. Further evaluation of causality and potential underlying mechanisms is warranted.

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A multicentre 25-year data on the performance of the 4-mg intravenous dexamethasone suppression test in the diagnosis of Cushing's syndrome

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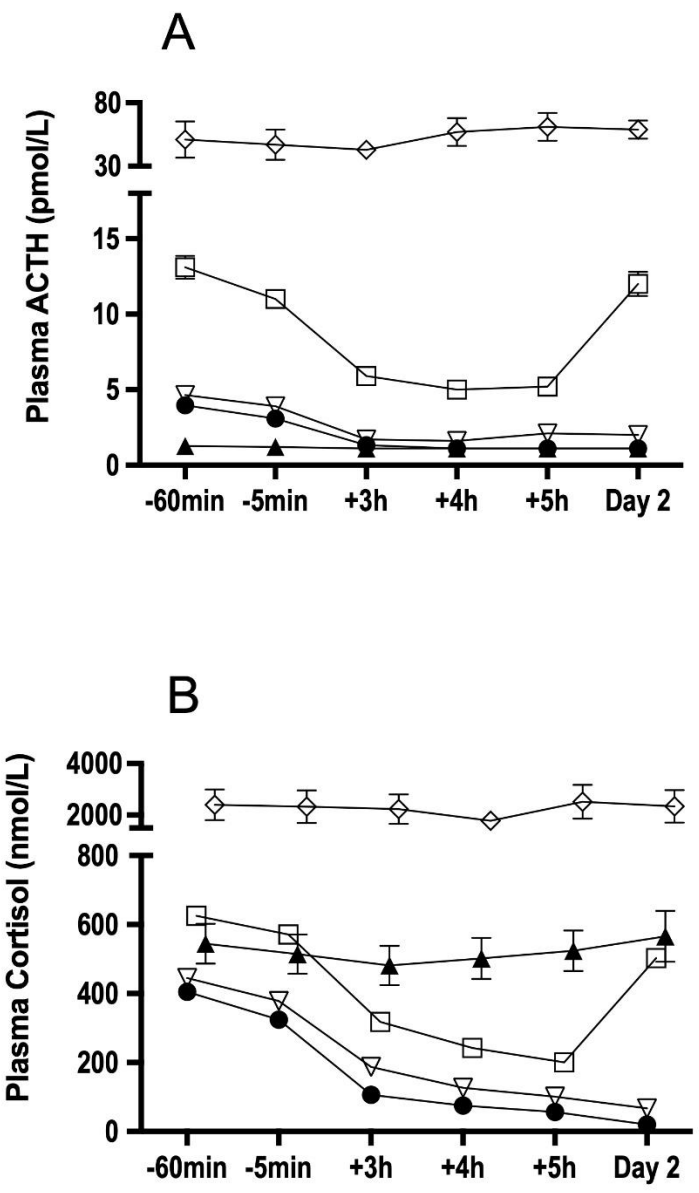
Mean (± SEM) ACTH (pmol/L) (A) and plasma cortisol (nmol/L) (B) during the IVDST in control group (●), LPC (▽), CD (□), AC (▲), and EAS (◇).



Objective: Differentiating Cushing's syndrome (CS) from Pseudo-Cushing's (Low Probability of Cushing's [LPC]) may be difficult. We evaluated the 4-mg intravenous dexamethasone suppression test (IVDST) to differentiate CS from normal subjects and LPC, and to define responses in CS of various causes.

Design: Data from 114 patients with Cushing's Disease (CD) who underwent IVDST(s) before their first pituitary operation (CDa), 27 patients who had repeat IVDST(s) after first pituitary operation (CDb), 22 with adrenal Cushing's (AC), four with ectopic ACTH syndrome (EAC) and 69 with LPC, from four tertiary hospitals between 1995 to 2020 were retrospectively evaluated. Thirty-two

control subjects (normal and overweight/obese participants with or without type 2 diabetes) were previously studied. Dexamethasone was infused at 1 mg/h for 4h. Plasma cortisol and ACTH were measured at -60 min (baseline), -5 min, +3h, +4h, +5h and at +23h and +23.5h on Day 2.

Results: Day 2 cortisol level of >130 nmol/L (or >30% of baseline) diagnosed CS with 99% sensitivity and 91% specificity. CDa group demonstrated partial suppression of cortisol, with rebound hypercortisolism on day 2 in 88% of patients. Six patients in CDb with suspected recurrence had day 2 cortisol levels <130 nmol/L. Cortisol levels did not suppress in AC or EAS. In 9 of 69 patients with LPC, day 2 cortisol overlapped with CS. Control subjects showed marked suppression of cortisol, maintained on Day 2.

Conclusion: IVDST has high sensitivity for diagnosis of CS. False negative results may occur in patients with surgically-proven CD when IVDST is performed during mild disease, quiescent phase, early recurrence, or with low volume ACTH-secreting pituitary tumour. The specificity of 91% is lower than previously reported (96%), highlighting the importance of long-term follow-up of LPC. Because only four EAC were studied, the utility of IVDST in differential diagnosis of ACTH-dependent CS is uncertain.

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The use of saline suppression test parameters to predict bilateral subtype of primary aldosteronism - an international multicentre study

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Background: The saline suppression test (SST) serves to confirm the diagnosis of primary aldosteronism (PA) while adrenal vein sampling (AVS) determines PA subtype. AVS however, is invasive, expensive and has limited availability. An accurate prediction of bilateral adrenal hyperplasia (BAH) based on SST could spare AVS in a significant cohort of patients. Our previous study suggested that a combination of plasma aldosterone concentration (PAC) <300 pmol/L and a reduced aldosterone-renin-ratio (ARR) following recumbent SST was 96.8% specific for predicting BAH in 121 patients [1].

Aim: To validate our criteria in an independent, international PA cohort, and to determine whether imaging characteristics, sex and potassium concentration might improve the accuracy of predicting BAH.

Methods: 289 patients were recruited (78 from Melbourne, Australia, 106 from Chongqing, China and 105 from Tokyo, Japan). Data including patient demographics, blood pressure, potassium supplementation and antihypertensive treatment; in addition to SST, adrenal imaging and AVS results were retrospectively analysed. Patients were subtyped based on AVS into unilateral aldosterone-producing adenoma (APA), BAH or indeterminate PA.

Results: At baseline, patients with BAH were more likely to be females with a lower PAC and higher serum potassium compared to those with APA in all cohorts. Following saline infusion, patients with BAH achieved a lower PAC compared to those with APA in all cohorts. Our criteria provided a specificity of 88.2%, 97.0% and 100.0% in the Australian, Chinese and Japanese cohorts respectively for predicting BAH. AVS could have been spared in 36.4%, 22.2% and 38.1% of BAH patients in the respective cohorts. Additional parameters including the absence of adrenal mass on CT, normokalaemia and female sex did not improve the predictive accuracy.

Conclusion: In recumbent SST, the criteria of PAC <300 pmol/L and a lower ARR post-saline are robust for predicting BAH in both Australian and Asian populations.

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Pre-operative mineralocorticoid receptor antagonist reduces postoperative hyperkalaemia in patients with Conn Syndrome

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BACKGROUND The pre-operative use of mineralocorticoid receptor antagonists (MRA) in patients with unilateral forms of primary aldosteronism (PA) is not consistent. Postoperative hyperkalaemia after adrenalectomy for PA management has been reported in up to 16% of patients (1,2). It is unclear whether pre-operative MRA can optimise peri-operative blood pressure and potassium control, and reduce the incidence of postoperative hyperkalaemia.

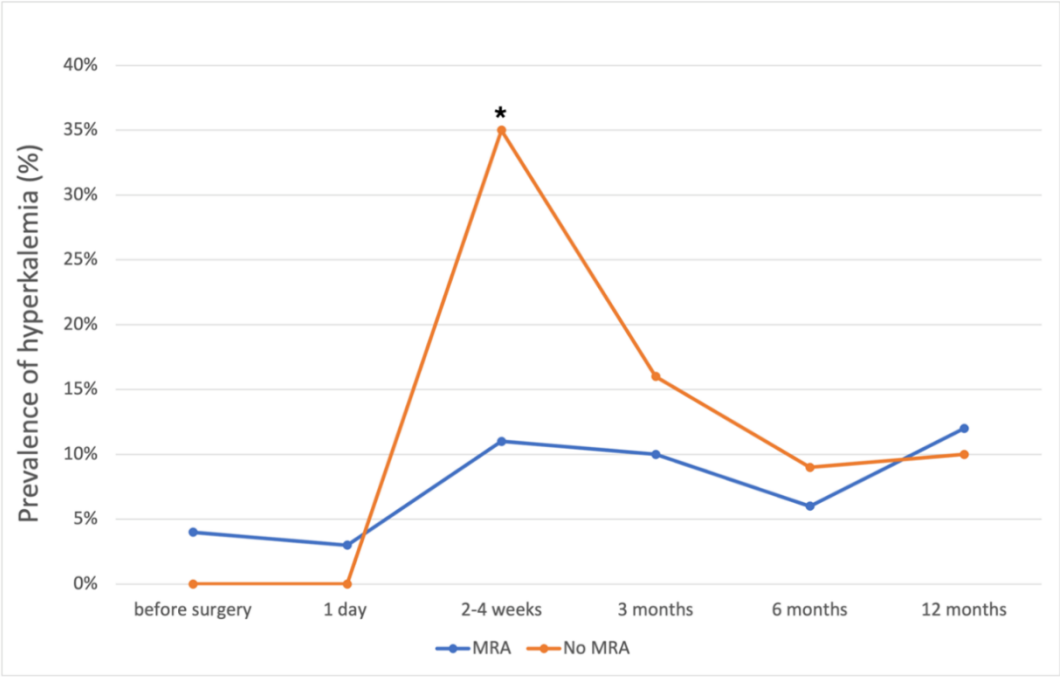
OBJECTIVE This study aimed to investigate the effect of pre-operative MRA on peri-operative blood pressure and potassium concentration, and the incidence of postoperative hyperkalaemia in patients undergoing unilateral adrenalectomy for the treatment of PA.

METHODS In this retrospective cohort study, a total of 96 patients with unilateral PA from a tertiary endocrine surgery database were included for analysis. Seventy-three patients ('MRA' group) received pre-operative MRA while 23 patients ('No-MRA' group) did not. The main outcome measures included the incidence of postoperative hyperkalaemia within 2 years after adrenalectomy, in addition to blood pressure and serum potassium concentration just prior to surgery.

RESULTS Spironolactone, an MRA, was administered at a median dose of 75mg for four months pre-operatively. The prevalence of postoperative hyperkalaemia was significantly higher in the 'No-MRA' group at 2-4 weeks after surgery, compared to the 'MRA' group (35% vs 11%, *p*=0.014, Figure 1). Logistic regression found the use of MRA to significantly predict a lower incidence of postoperative hyperkalaemia after adjusting for age, sex, baseline aldosterone-to-renin ratio and potassium concentration (b=-2.029, *p*=0.012). Prior to surgery, patients in the 'MRA' group had normalised blood pressure and potassium concentration with fewer antihypertensive medications and no potassium supplements.

CONCLUSION Pre-operative MRA use was associated with optimal perioperative blood pressure and normalised serum potassium in addition to a lower incidence of postoperative hyperkalaemia. MRA should be considered standard treatment for patients awaiting surgery for PA.

Figure 1: The prevalence of postoperative hyperkalaemia in the 'MRA' and 'No-MRA' groups



Hyperkalaemia is defined as serum potassium >5.0 mmol/L. Timepoints include immediately before surgery, postoperative 1 day, 2-4 weeks, 3 months, 6 months, and 12 months. *p <0.05

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Cost-effectiveness of screening for primary aldosteronism in hypertensive patients

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Introduction:

Primary aldosteronism (PA) affects 3%-13% of hypertensive patients in the primary care setting and up to 30% in the hypertensive referral units. Although the Endocrine Society Guidelines recommend screening for PA in patients with severe or treatment-resistant hypertension, the diagnosis of PA at an earlier stage of the disease has the potential to prevent end-organ damage and optimise patient outcomes.

Objective:

This study aimed to estimate the cost effectiveness of screening for PA in treatment- and disease (cardiovascular disease and stroke)-naïve hypertensive patients.

Methods:

A Markov Model was used to compare the costs and effectiveness of screening for PA. Within the model, a 40-year-old patient with hypertension went through either the screened or unscreened arm of the model and was modelled until age 80 or death. In the screening arm, the patient underwent screening and standard diagnostic testing for PA. The main outcome of interest was the Incremental Cost Effectiveness Ratio (ICER) and a willingness to pay threshold of AU$50,000 was used.

Results:

Screening hypertensive patients for PA as compared to not screening attained an ICER of AU$21,768 per quality-adjusted life year gained. The results were robust to different sensitivity analyses, in particular, screening was cost-effective irrespective of the discount rate and duration of follow-up beyond 5 years.

Discussion and Conclusion:

The results from this study demonstrated that screening all hypertensive patients for PA from age 40 is cost-effective because the ICER is lower than the willingness to pay threshold. The data suggest that screening for PA should be performed before the development of severe or treatment-resistant hypertension in the Australian healthcare setting.

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Low blood levels of testosterone and DHEA are associated with an increased risk of ischemic cardiovascular events in older women

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Background

Whether higher circulating testosterone is favourable or disadvantageous for ischemic cardiovascular risk and total mortality in older women is not known

Methods

Australian women, aged 70+ years, with no prior cardiovascular disease events, dementia, or physical disability were recruited between 2010 and 2014. The primary endpoint for this study was major adverse cardiovascular events (MACE): fatal coronary heart disease (excluding heart failure), nonfatal myocardial infarction, and fatal or nonfatal ischemic stroke. All-cause mortality was examined. Sex steroids were measured by liquid chromatography, tandem mass spectrometry.

Results

Of the 9187 Australian female participants, 6392 provided biobank samples and 6358 had sex steroid measured. The included women were aged 70 to 94·8 years, median (IQR)= 73.9 (5.8). Most (98·9%) were of European ancestry, 66% had two or more cardiovascular disease risk factors and 31% were obese. After a median of 4.5 years of follow-up (28,187 person-years) 158 of the 6358 women experienced a first ever MACE, an incidence rate of 5.6/1000 person-years. After adjusting for likely confounders including the number of known cardiovascular disease risk factors, the hazard ratio (HR) for women in the 4th (highest) quartile of testosterone compared with the 1st (lowest) quartile was 0.53 [95% CI, 0.34-0.81, p=0.004]. The HR for Q3 was significantly different from Q1 (p=0.03) and Q2 approached significance (p=0.06). Compared with Q1, all other DHEA quartiles were associated with significantly lower risk of MACE in the fully adjusted models. Sex steroid concentrations were not significantly associated with all-cause mortality.

Conclusions

Testosterone and DHEA concentrations above the lowest quartile in older women are associated with a reduced risk of a first ever MACE. As the physiological effects of DHEA are mediated through its steroid metabolites, should these findings be replicated, primary prevention trials of testosterone to prevent MACE in older women would be warranted.

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Diagnosis of 21-hydroxylase deficient non-classic congenital adrenal hyperplasia and heterozygosity by liquid chromatography and tandem mass spectrometry

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NCCAH is a key differential diagnosis for PCOS. NCCAH is diagnosed via the SST when 17OHP is >30 nmol/L. Increasing use of LC-MS/MS necessitates revision of this immunoassay-derived threshold. Heterozygotes (HTZ) may present for investigation of hyperandrogenic symptoms [1]. As 17OHP is normal in 20-70% of HTZ, diagnosis relies on expensive molecular studies. A biochemical test for HTZ would economise use of molecular studies and support genetic counselling when genotyping is unavailable. 21-deoxycortisol (21-DF) may be useful for HTZ diagnosis[2-4].

We aimed to define LC-MS/MS-specific criteria for NCCAH and HTZ.

From Pathwest QEII laboratory database, we identified genotyped females>15yrs who had undergone an $SST_{LC\text{-}MS/MS}$ from January 2010 to June 2017, and prospectively recruited hyperandrogenic females referred for an SST from June 2017 to August 2021. Steroids were compared among genetically confirmed PCOS, HTZ and NCCAH.

17 $OHP_{LC\text{-}MS/MS}$ results were available for n=81 (53 PCOS, 19 HTZ and 9 NCCAH). 21-DF was also estimated in n=36 (21 PCOS, 9 HTZ and 6 NCCAH). The best single parameter to discriminate HTZ from PCOS was $21\text{-}DF_{30mins.}$

Diagnostic thresholds to distinguish HTZ (plus NCCAH) from PCOS

Parameter	Optimal Threshold	sensitivity	spec
$21\text{-}DF_{30mins}$	1.01 nmol/L	100%	85.7
17 OHP_{peak}	8.05 nmol/L	100%	81.1
(21DF+17OHP)/$cortisol_{60mins}$x1000	13.6 (unitless)	100%	88.2

Diagnostic thresholds for NCCAH

Parameter	Optimal Threshold	sensitivity	spec
Basal 17 OHP	3.55 nmol/L	93.3%	88.6
17 OHP_{peak}	20.7 nmol/L	100%	98.6
Basal 21-DF	0.31 nmol/L	100%	96.7
$21\text{-}DF_{peak}$	13.31 nmol/L	100%	100

Conclusion: ACTH-stimulated 21-DF and 17OHP measured by LC-MS/MS permit excellent discrimination between HTZ and PCOS. Thresholds for NCCAH are lower by LC-MS/MS than those defined by immunoassays.

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T4DM Assays and Run-off

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Introduction of T4 Bone

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miR-23b-3p is involved in regulating human endometrial receptivity implying a role in implantation

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The human endometrium undergoes dramatic remodelling and molecular changes throughout the menstrual cycle that are essential for successful blastocyst attachment in the receptive, mid-secretory phase. microRNA (miR) levels are altered in the receptive phase and are thought to play a role in blastocyst implantation. miR-23b-3p is known to be differentially expressed in the endometrium of women with normal fertility compared to infertility. We aimed to investigate the expression of miR-23b-3p in human endometrium across the menstrual cycle, its function, and mechanisms of action in receptivity. Using qPCR, we demonstrated that miR-23b-3p was significantly upregulated in the fertile endometrium of the mid-secretory (receptive) compared to proliferative (non-receptive) phase ($p<0.05$). miR-23b-3p was identified in both the epithelium and stroma, with highest expression found in the endometrial epithelium during the mid-secretory phase. Ishikawa cells (endometrial epithelial cell line) and primary endometrial epithelial cells (EEC) were used to determine the effect of miR-23b-3p overexpression on adhesive capacity to trophectodermal spheroids. Overexpression of miR-23b-3p improved the adhesive capacity of endometrial epithelial cells ($p<0.05$). miR-23b-3p predicted mRNA targets (n=11) were investigated using qPCR and MET, SFRP4 and ACADSB were significantly downregulated in miR-23b-3p transfected EEC compared to scramble control ($p<0.05$). Mass spectrometry analysis was performed comparing Ishikawa cells overexpressing miR-23b-3p and control. 59 proteins were differentially expressed ($p<0.05$). STRING network analysis revealed significant protein interactions (protein-protein enrichment p-value<0.05), with functional enrichment in focal adhesion, integrin-mediated cell adhesion, cell-substrate junction pathways. Of the 59 differentially expressed proteins, 9 were predicted miR-23b-3p targets in the miRWalk database. Our data demonstrated that miR-23b-3p is likely a critical regulator of epithelial adhesion and endometrial receptivity. miR-23b-3p could serve as a diagnostic to identify which women may benefit from personalised therapeutics, specifically that target miR-23b-3p to improve receptivity, implantation potential and the establishment of pregnancy.

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High-throughput in vitro screening identified Nemadipine-A that suppressed embryo implantation in vitro and in vivo

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Nowadays, different approaches are used to inhibit follicular development prevent ovulation, fertilization and embryo implantation for contraception. Hormonal contraceptives are commonly used to inhibit ovulation but are associated with many side effects. To identify novel non-hormonal compounds as contraceptives to prevent embryo implantation, we established a high-throughput human spheroid-endometrial epithelial cell co-culture model to screen the Library of Pharmacologically Active Compounds (LOPAC) for small molecules that affect the attachment of trophoblastic spheroids (blastocyst surrogate) onto endometrial epithelial cells (endometrium surrogate). A total of 174 out of 1280 LOPAC compounds significantly suppressed BeWo spheroid attachment onto endometrial Ishikawa cells. One of the top 20 active compounds (P11B5, Nemadipine-A) having the lowest cytotoxicity in Ishikawa cells was selected for further study. Nemadipine-A at 10 μM also significantly suppressed the attachment of BeWo spheroids onto endometrial epithelial RL95-2 cells (41.3 vs 52.3 %, $p<0.01$) and primary human endometrial epithelial cells (hEECs, 6.5 vs 17.1%, $p<0.01$) collected on LH+7/8 days. The suppressive effect on embryo implantation was also observed after transcervical injection of Nemadipine-A (100 μg/kg) or Misoprostol (250 μg/kg) but not Nemadipine-A at 10 μg/kg into mouse uterine cavity on 1.5 days post coitum (dpc) when compared with the controls. The expressions of endometrial receptivity markers integrin aV (ITGAV), mucin-1 (MUC1) but not b-catenin (CTNNB1) transcripts were statistically reduced on 2.5 dpc when compared with the untreated control. In sum, LOPAC screening identified Nemadipine-A as an inhibitor of spheroid attachment onto Ishikawa, RL95-2 cells and primary hEECs. The suppressive effect on mouse embryo implantation of Nemadipine-A may work through regulating endometrial receptivity molecules ITGAV and MUC1. Nemadipine-A can be a potential non-hormonal

contraceptive drug inhibiting the implantation process. [Supported grants: the Sanming Project of Medicine in Shenzhen, China (SZSM201612083), Shenzhen Key Medical Discipline (SZXK2020089), General Research Fund, Research Grants Council, Hong Kong (17120720)]

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Role of the prorenin receptor in endometrial cancer cell growth

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Endometrial cancer is the most commonly diagnosed gynecologic malignancy in women after breast, lung and colorectal cancer. Despite numerous scientific advances, the incidence and mortality rate of endometrial cancer continues to rise. Considerable research effort has been placed on understanding the pathogenesis of this disease. Emerging evidence now suggests a putative role of the (pro)renin receptor ((P)RR), in the ontogenesis of endometrial cancer. Support for this notion arises from literature implicating the (P)RR in breast cancer and pancreatic carcinoma pathophysiology by virtue of its role in proliferation, angiogenesis, fibrosis, migration and invasion.

In view of these data, we aimed to investigate the functional role of the (P)RR in human endometrial cancer progression and development. To this end, we employed an siRNA-mediated knockdown approach to abrogate (P)RR expression in the immortalized endometrial epithelial cell lines; Ishikawa, AN3 CA and HEC-1-A and examined cellular proliferation and cellular viability. To further extend these analyses we also carried out a sophisticated proteomic screen, to explore potential pathways via which the (P)RR is acting in endometrial cancer physiology. These data confirmed that the (P)RR is critical for endometrial cancer development, contributing to both its proliferative capacity and in the maintenance cell viability. This is likely mediated through proteins such as MGA, SLC4A7, SLC7A11 or DHRS2, which were reduced following (P)RR knockdown. These putative protein interactions/pathways, which rely on the presence of the (P)RR, are likely to contribute to endometrial cancer progression and could therefore, represent several novel therapeutic targets in the treatment of this cancer.

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Altered epigenetic programming of oocytes modulates offspring growth and accelerates bone development

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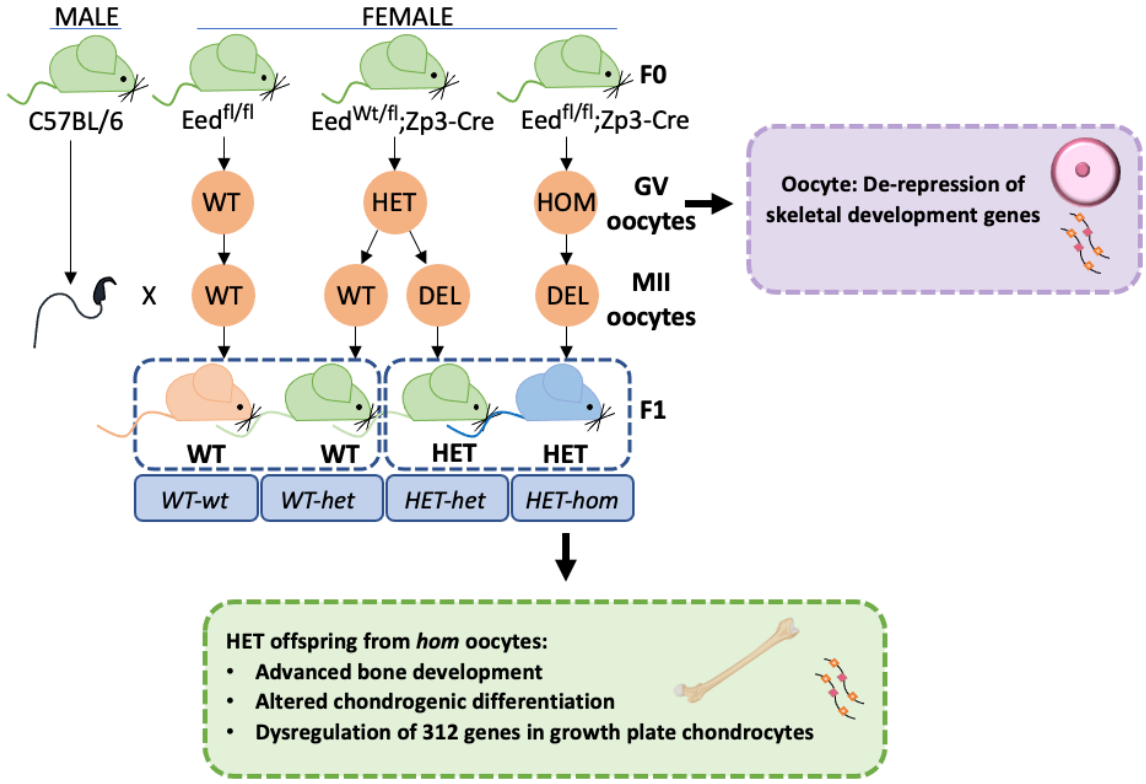
Epigenetic programming in the germline is considered to affect development in offspring, but the mechanisms are poorly understood. Embryonic Ectoderm Development (EED) is essential for Polycomb Repressive Complex 2 (PRC2) function, which epigenetically regulates developmental genes in bone. PRC2 is a known regulator of bone stem cell differentiation and is implicated in maintenance of adult bone health. *De novo* germline mutations in human EED result in Cohen-Gibson syndrome, characterized by overgrowth, accelerated bone aging and skeletal defects. However, EED's potential to alter oocyte epigenetic programming and consequent offspring development is poorly understood.

To determine the role of EED in oocyte programming, we developed a mouse model in which *Eed* is specifically deleted in growing oocytes. This model facilitates the production of genetically identical heterozygous offspring from oocytes with differences in their epigenetic heritage. We predicted that offspring from oocytes lacking EED would have impaired bone development, as well as compromised bone maintenance and repair.

Loss of EED in oocytes resulted in the de-repression of 244 genes (FDR<0.05) that were primarily associated with fetal development, including bone formation. MicroCT analyses demonstrated that postnatal day 3 offspring from oocytes lacking EED exhibited greater bone mineral density, increased mineralised bone length ($p<0.05$, n=8-10) and bone width ($p<0.0005$, n=8-10). Histological and RNAseq analyses revealed that loss of EED in oocytes resulted in an increased hypertrophic zone ($p<0.05$, n=8-12) and dysregulation of 312 genes (FDR<0.05) in E17.5 femoral growth plates, indicative of abnormal transcriptional regulation and chondrocyte differentiation in heterozygous experimental offspring, compared to genetically identical heterozygous controls.

Together, these data strongly suggest that altered EED-dependent oocyte programming results in postnatal overgrowth and altered bone development, including similarities to skeletal defects associated with Cohen-Gibson syndrome. This model will be

used to identify how inherited epigenetic information controls early life and long-term skeletal development and health.



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Polycomb-dependent epigenetic programming in the oocyte modulates brain development and behaviour in offspring

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Epigenetic information transmitted by eggs and sperm is thought to affect offspring development, but the mechanisms involved are poorly understood. Determining the molecular basis for epigenetic inheritance is critical given that the germline activity of epigenetic modifying enzymes can be altered by environmental influences, such as diet and drugs, with subsequent impacts on inheritance.

Embryonic Ectoderm Development (EED) is a core component of Polycomb Repressive Complex 2 (PRC2), which is a highly conserved epigenetic regulator required in oocytes for growth and development in offspring. PRC2 catalyses tri-methylation of lysine 27 in histone 3 (H3K27me3), thereby repressing developmental genes in multiple tissues.

To determine the role of PRC2 in programming inherited impacts on brain development and behaviour, we deleted EED only in growing mouse oocytes and analysed outcomes in offspring. This model enabled the study of epigenetic inheritance through the production of genetically identical offspring from eggs that lacked EED-dependent epigenetic programming. Consistent with a role for EED-dependent maternal programming, oocytes lacking EED had severely depleted H3K27me3 and an extensive range of neurodevelopmental genes were de-repressed. Moreover, histological and immunofluorescence analyses of key neurological markers in the developing brain revealed significant neurological developmental delay in embryonic offspring from oocytes in which *Eed* was deleted. Additionally, these offspring had significantly reduced brain weight at birth compared to genetically identical controls ($n=59$, $p<0.005$), indicative of altered brain development, and current RNA sequencing of developing cortex is expected to identify pathways that are dysregulated. Extensive behavioural testing in adult mice revealed that offspring from oocytes lacking EED had a severely blunted response to the challenge drug methamphetamine, compared to genetically identical control offspring ($n=113$, main effect of drug: $p<0.001$).

These data provide evidence that EED-dependent epigenetic programming in the oocyte plays an essential role in regulating non-genetic inheritance of brain development and behaviour.

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Human endometrium derived mesenchymal stem cells and exosome: understanding their phenotype for therapeutic prospects

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Background: Human endometrium harbors a rare population of SUSD2^{+} perivascular/highly clonogenic mesenchymal stem cells (eMSC) that can be harvested as an office-based procedure without anaesthesia.

Aim: To define the in vitro secretory and phenotype profile eMSC that can predict their mechanism of action and functional behaviour in vivo, and to determine eMSC-derive exosomes-cargo and their therapeutic potential.

Methods: SUSD2^{+} eMSC were expanded in serum-free media/TGF-βR inhibitor (A83-01). Exosomes were isolated from the conditioned medium using differential ultra-centrifugation. They were characterised using Western blot analysis for exosome markers, ZetaView® for size analysis, particle count and transmission electron microscopy (TEM) for morphology. Quantitative mass spectrometry was performed to identify exosome cargoes and differential protein content. eMSC/A83-01 were also primed with inflammatory cytokines TNF-α and IFN-g for 72 hours. Conditioned media and cells were assessed for secretory and surface expression phenotype.

Results and conclusions: Our comprehensive protein cargo analysis of eMSC exosomes showed that eMSC/A83-01 secreted a wide range of pro-angiogenic, anti-fibrotic molecules and anti-inflammatory cytokines compared to untreated eMSC. sEV expressing Alix, TSG101 and Syntenin-1 with ~120nm and central depression were identified. eMSC/A83-01 had low immunogenicity before and after exposure to inflammatory cytokines with no expression of HLA-DR and CD86 but high expression of immunomodulatory molecules HLA-ABC, CD200 and CD274. eMSC are at immune haemostasis and only secreted IDO and PGE2 after priming with inflammatory cytokines demonstrating dampening effect on multiple immune cells including T-cell proliferation and NK-mediated apoptosis. In addition, eMSC/A83-01 had significantly decreased CD142 expression, indicating a low procoagulant effect however, this increased after inflammatory activation indicating a pro-angiogenic effect in an inflammatory environment. In conclusion, A83-01-treated eMSCs have enhanced therapeutic properties, suggesting their broad potential for allogenic cell-therapies or cell-free exosomes in regenerative medicine.

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Investigating a role for the hypoxia-inducible factor EPAS1 in spermatogonial stem cell function

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Spermatogonial stem cells (SSCs) reside within an 'open niche' in the testis that emanates key regulatory signals to balance self-renewal versus differentiation. It has been proposed that vicinity to testicular vasculature (and thus oxygen availability) may influence SSC fate, as is the case multiple stem cell niches. In support of this, our analysis of single cell RNA-sequencing (scRNA-seq) data produced from postnatal day 6 mouse spermatogonia revealed enrichment of hypoxia-inducible transcription factors (HIFs) in the SSC population (*Gfra1*+, *Id4*+). Contrastingly, normoxia-responsive factors that tag HIF proteins for degradation were enriched in progenitor (*Neurog3*+, *Sox3*+) and differentiating (*Stra8*+, *Kit*+) spermatogonia. We elected to further characterise HIF2A ("EPAS1") expression using the *Id4-eGfp* transgenic mouse to identify SSCs (GFP-'Bright' cells). Using immunofluorescence techniques, over 90% of SSCs were found to express EPAS1, compared to <10% of progenitor spermatogonia (P<0.001). Similarly, when *Id4-eGfp* mice were injected with 'hypoxyprobe' (pimonidazole), hypoxia-associated protein adducts were identified in >80% SSCs compared to <10% progenitor spermatogonia (P<0.001). To determine a functional role for EPAS1 in SSC regulation, a germline-specific *Epas1*-knockout mouse line was produced ("*Epas1*-cKO"). *Epas1*-cKO males are fertile up to 6 months of age (breeding study ongoing), however quantitative differences in spermatogenesis are apparent. Notably, at 6 months, *Epas1*-cKO testis weight is increased by 10%, epididymis weight by 30%, and caudal sperm concentration by 10%, when compared to control animals (n=1). Contrastingly, sperm motility and viability are decreased by 65% and 40%, respectively. Quantitative differences in sperm production in *Epas1*-cKO mice may suggest perturbation of mitosis in slow-cycling SSCs, or a shift in the SSC-progenitor equilibrium: possibilities that are currently under investigation. These data suggest that at least a subset of SSCs preferentially reside in hypoxic microenvironments in the testis, and that HIFs may influence cell fate decisions and/or mitotic regulation in these cells.

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Anti-viral defences in the male reproductive tract: Expression and localisation of the novel type I interferon epsilon

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Interferon epsilon (Ifnε), a novel type-I interferon constitutively expressed in female reproductive epithelia, protects female mice against sexually transmitted viruses. Production, cellular localization, and functions of Ifnε in the male reproductive tract have not been studied previously. Since many viruses, including HIV, HSV2, HepB, mumps, Zika, and SARS disrupt male reproductive function, investigation of this potent anti-viral agent in the male was warranted.

Expression and localization of Ifnε in the male reproductive tract was studied by indirect immunofluorescence and qRT-PCR in 25 and 56 day old wild-type mice relative to *Ifnε*$^{-/-}$ mice (n = 8/genotype). Wild-type testes between days 5 and 180 were examined to determine *Ifnε* expression during postnatal development. Cellular sites of production were determined in individual testicular cell types isolated from 44 day old mice using Percoll gradient centrifugation, centrifugal elutriation and lectin-adherence.

Ifnε was differentially expressed within the male reproductive tract. Expression was very high in the testis, absent in the epididymis, and low in the vas deferens at both day 25 (prior to the completion of sperm production), and day 56 (mature spermatogenesis). Testicular *Ifnε* first appeared between day 20 and 25, and was expressed in meiotic and post meiotic germ cells, and in interstitial cells, particularly macrophages. Interferon-stimulated genes (ISG), such as *Isg15*, *Irf7,* and *Oasl2*, displayed low expression in the testis, and gradually increased towards the cauda epididymis and vas deferens, in parallel with other type-I interferons, such as *Ifnb1*. Unlike in the female, *Ifnε* deficiency did not reduce ISG expression within the male reproductive tract.

Constitutive expression of Ifnε in the mature testis, the region most prone to viral infections within the male reproductive tract, suggests a crucial role for Ifnε in testicular innate immunoregulation and protection, whereas other type-I interferons may play a more significant protective role in the distal epididymis.

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The canonical Katanin complex is essential for fertility during early embryonic mitoses but not in meiosis

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Microtubule dynamics are essential for the production of a normal meiotic spindle and fertile oocyte. Microtubule severing by the Katanin protein family has been identified as a means of regulating microtubule function. Katanin is an evolutionarily conserved microtubule-severing complex consisting of Katna1, a severing enzyme and Katnb1, a regulatory subunit. The aim of this study was to investigate the role of katanin-mediated microtubule severing in meiotic and mitotic divisions of the oocyte and early mouse embryo.

We developed two conditional knockout mouse models by crossing Katna1 or Katnb1-floxed mice with Zp3-Cre mice. Breeding experiments show infertility in the Katnb1$^{(-/-)}$, and subfertility in the Katna1$^{(-/-)}$ mice. Focusing on the Katnb1$^{(-/-)}$, we saw no difference in the first meiotic spindles compared to controls but at metaphase II, there was a significant increase in spindle abnormalities (20.0% vs 37.0%; $p<0.05$). After IVF with wildtype sperm to create heterozygous embryos, there was no significant effect on the rate of 2-cell or blastocyst formation, however blastocysts were significantly smaller, with fewer cells (49.5 vs 17.6; $p<0.0001$) which had a 100% increase in nuclear volume. To examine the role of Katnb1 in the absence of the paternal allele, diploid homozygous Katnb1$^{(-/-)}$ embryos were generated by parthenogenetic activation. The complete absence of Katnb1 resulted in only18.3% of 1-cell embryos reaching the blastocyst stage compared to 79.2% of controls ($p<0.01$), and the blastocysts generated were morphologically abnormal, with very low cell numbers (71.7 vs 11.5; $p<0.0001$) and a 100% increase in average nuclear volume. Time-lapse imaging revealed a high incidence of mitotic failure, asymmetric cell division in the first few mitotic cell divisions of Katnb1$^{(-/-)}$parthenogenetic embryos.

In summary, Katnb1$^{(-/-)}$ is essential for fertility and plays critical roles in spindle formation and function during MII and the early embryonic cell divisions.

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Prenatal Alcohol Exposure and Mental Illness

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High dose alcohol consumption during pregnancy increases the risk for a plethora of adverse offspring outcomes. Both clinical and pre-clinical studies have demonstrated prenatal alcohol exposure can contribute to offspring with neurodevelopmental, cognitive and social deficits, as well as psychiatric illnesses, such as depression and anxiety. However, much less evidence is available on the effects of low dose and alcohol exposure in early pregnancy on mental health outcomes. This is critically important as many women have unplanned pregnancies and are consuming alcohol prior to pregnancy recognition.

We have developed a rodent model of moderate prenatal alcohol exposure that is confined to time around conception. Both male and female offspring exposed to this 'periconceptional' alcohol (PC:EtOH) developed altered neuroendocrine function and behaviour. PC:EtOH exposure resulted in a significant increase in depressive- and anxiety-like behaviours as well as altered social phenotypes. Interestingly, PC:EtOH exposure reduced basal plasma corticosterone concentrations in female but not male offspring. In early life, there was no impact of PC:EtOH on plasma corticosterone responsiveness however, in aged offspring, stress-induced plasma corticosterone and the pressor response to restraint were significantly reduced in female offspring. Adrenal and hypothalamic mRNA expression of genes regulating glucocorticoid production were not overtly altered by PC:EtOH. However, aged female offspring exposed to PC:EtOH, demonstrated increased expression of the glucocorticoids receptor in the hippocampus, suggesting altered hypothalmic-pitutary-adrenal (HPA) regulatory pathways.

This study supports the hypothesis that alcohol exposure even prior to implantation programs sex-specific alterations in offspring in a rodent model. Effects of alcohol on the maternal HPA and related physiological changes as a consequence of PC:EtOH are likely to underlie behavioural outcomes observed in this study. This research highlights the importance of public health messaging recommending abstinence from alcohol as part of preconception planning.

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Prenatal Androgen Excess Impairs Sexual Behavior in Adult Female Mice: Perspective on Sexual Dysfunction in PCOS

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Polycystic ovary syndrome (PCOS) is the most common infertility disorder worldwide, affecting 5-20% of reproductive aged women [1]. PCOS is characterised by high circulating androgen levels, oligo- or anovulation, and polycystic ovarian morphology [1]. PCOS patients also experience sexual dysfunction, such as decreased sexual desire, increased sexual dissatisfaction and gender dysphoria [2-4]. The origins of these sexual difficulties remain unidentified. The well-characterized prenatally androgenized (PNA) mouse model of PCOS exhibit an adult hyperandrogenism, impaired sex steroid feedback and alterations in the neuronal network regulating reproductive function [5]. Thus, the PNA model of PCOS provides a powerful, pathology-based model to unravel a potential biological origins of sexual dysfunction in PCOS. We hypothesized that the PNA mouse model of PCOS will exhibit an impairment of female sexual behaviours. To model PCOS, female dams received injections of dihydrotestosterone (PNA) or oil vehicle (VEH) daily from gestational day 16 to 18. Adult female offspring were ovariectomized and implanted with a silastic capsule of estradiol to examine the female-typical sexual behaviour, lordosis; partner preference and male-like sexual behaviour. PNA females exhibited an overall reduction in lordosis behaviour compared to VEH females ($p<0.01$). Interestingly, partner preference and male-like sexual behaviour weren't altered in PNA compare to VEH females. These results show for the first time that prenatal androgen exposure impairs sexual function. Neuronal activation after sexual behaviour is mainly not affected by prenatal androgen excess except in one part of the brain: the dorsomedial hypothalamus where a decrease in cFos immunoreactivity has been observed. Current experiments are underway to determine the neuronal target of prenatal androgen exposure implicated in the central control of lordosis. Taken together, this study open novel perspective on the origins of sexual dysfunction in women with PCOS and further investigation still remains to understand the biological mechanism of prenatal androgen exposure on the female brain and sexual function.

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Metabolic Sensing of Food Availability Integrates Hunger with Motivation and Adaptive Behaviour

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The motivation to seek palatable, energy-dense food evolved as a key mechanism for survival and maturation in an environment with limited food availability. Given that heightened motivation for palatable food in an environment of low food availability shaped an evolutionary benefit, it is not surprising that neural circuits controlling and responding to body weight have a profound effect on motivation and adaptive behaviour. We are particularly interested in how the brain senses low food availability to appropriately control motivated and adaptive appetitive behaviours, such as increase exploration and reduced anxiety. An understanding of these processes may offer insight into the pathogenesis of obesity or altered behavioural control as seen in eating disorders. The peripheral hormone ghrelin and its key neural target population, Agouti-related peptide (AgRP) neurons, are critical components of the body's response to hunger and low food availability. Ghrelin is often referred to as a hunger-signalling hormone and AgRP neurons are often referred as hunger-sensing neurons. In this talk I will discuss how AgRP neurons and ghrelin receptor-expressing neurons respond to hunger and influence behavioural responses to ensure adequate food consumption. This includes the impact on motivated behaviour through dopamine signalling and adaptive behaviours involved in mood control.

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The Role of Microglia in Regulating Satiety and Cognition

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Microglia have myriad roles in the central nervous system additional to their immune ones. However, their functions in the healthy adult remain poorly understood. To understand how microglia contribute to regulating satiety, feeding and cognitive function in healthy individuals, we developed a Wistar rat with a diphtheria toxin receptor in the promoter region for the fractalkine receptor (Cx3cr1), which is expressed on microglia and monocytes. This model allows acute microglial and monocyte ablation (followed by repopulation) upon application of diphtheria toxin, enabling us to directly assess microglial function. Short-term microglial ablation in these rats leads to a dramatic weight loss that is largely accounted for by an acute reduction in food intake, with these rats showing a particular aversion to highly palatable food. This weight loss and anorexia are not likely due to a sickness response since the rats do not display peripheral or central inflammation, withdrawal, anxiety-like behavior, or nausea-associated pica. Hormonal and hypothalamic anatomical changes are largely compensatory to the suppressed food intake, which occurs in association with disruption of the gustatory circuitry at the paraventricular nucleus of the thalamus. With this model, we also show that cognition in the novel object and place recognition tasks is entirely unaffected by acute microglial ablation. However, when microglia repopulate the brain, learning and memory performance in these tasks is improved. This transitory cognitive enhancement is associated with an ameboid morphology in the newly replaced microglial cells as well as with differences in synaptic markers and the density of mature hippocampal synaptic spines. Together these data indicate that microglia are important for supporting normal feeding behaviors, as well as learning and memory. They suggest these cells may be a useful potential target for satiety control and to strategically enhance memory; and that our understanding of microglia needs to be expanded.

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Multiscale analysis of single cell transcriptomics, human genetic association studies, and knockout mouse models identifies genes and cell types associated with bone mineral density and height

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Background: Bone mineral density (BMD) is a major determinant of bone strength and adult height reflects the outcome of skeletal growth. Genome-wide association studies (GWAS) have identified hundreds of genomic regions associated with BMD and height, however the underlying genes and cell types remain unclear. We hypothesised that integration of GWAS, single cell RNA sequencing (scRNA-seq) data and knockout mouse (KO) models would identify genes associated with BMD and height in a cellular context.

Methods: scRNA-seq was used to map the transcriptome of cells isolated from mouse femurs. Hypergeometric tests were used to identify cell types enriched for genes involved in monogenetic skeletal disorders. MAGMA gene-set analysis was used in conjunction with GWAS of 448,010 participants in the UK-Biobank Study to identify cell types that are enriched for BMD- and height-associated genes. Skeletal phenotyping of 1000 KO mouse lines was used to validate the functional roles of identified genes.

Results: scRNA-seq analysis of 133,942 cells identified 34 cell types with distinct transcriptional profiles. Osteoblasts and osteoclasts were enriched for genes that cause monogenetic low and high bone mass skeletal disorders respectively, whereas chondrocytes were enriched for genes involved in disorders of bone growth ($P<3\times10^{-7}$). MAGMA identified 3681 BMD- and 7382 height-associated genes ($P<2.5\times10^{-6}$). Osteoblasts, endothelial and vascular smooth muscle cells were enriched for BMD-associated genes, whereas chondrocytes were enriched for height-associated genes ($P<5\times10^{-4}$). In-depth phenotyping of 1000 unselected KO mouse lines showed that cell type specific BMD- and height-associated genes are more likely to result in skeletal abnormalities when deleted in mice ($P<1\times10^{-3}$). Novel BMD-associated genes were identified, including the endothelial-specific gene *Slc9a3r2*, which when deleted in mice resulted in reduced trabecular bone mass.

Conclusions: Our multiscale approach identified novel BMD- and height-associated genes, and the cellular context through which they may function to regulate bone homeostasis and growth.

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Transcriptional mapping of the endosteal dormant tumour cell niche

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Despite advances in the treatment of primary cancers skeletal metastases remain a major cause of disease relapse and mortality. Within bone, metastatic relapse results from the dissemination, long-term survival and eventual reactivation of dormant tumour cells (DTCs). DTCs from many common cancers are found associated with cells neighbouring the endosteum, through a mechanism that currently remains unclear. We hypothesised that tumour cell dormancy is controlled by specific interactions between disseminated cancer cells and cells of the endosteal bone compartment.

Using murine models of bone metastasis, we isolated both dormant and reactivated myeloma, breast and prostate cancer cells and performed single-cell sequencing to identify genes upregulated during dormancy. In parallel, we sequenced 133,942 cells from the endosteal and marrow compartments, delineating 34 endogenous bone microenvironment cell types. Using this bone cell map, we performed a ligand-receptor analysis to predict microenvironment crosstalk with DTCs, for myeloma, breast and prostate cancer.

Across all cancer models, endosteal osteoblasts were most enriched for genes predicted to interact with DTCs (*adjusted p values* – myeloma: $2.1x10^{-6}$, breast: $1.1x10^{-21}$, prostate: $1.4x10^{-24}$). Trajectory analysis of the osteoblast lineage revealed that DTC crosstalk enrichment was highest in $Cxcl12^{high}$-$Lepr^{high}$ mesenchymal stromal cells (MSCs) (myeloma: $7.6x10^{-9}$, breast: $3.0x10^{-11}$, prostate: $9.5x10^{-24}$). Comparison between cancers identified potential pan-cancer dormancy regulators, including MSC-derived Gas6 binding to TAM receptors (Tyro3/Axl/Mertk) on DTCs which, when inhibited *in vivo*, released myeloma cells from dormancy. A $Cxcl12^{high}$-$Lepr^{high}$ MSC population was identified expressing DTC crosstalk genes (including Gas6) within the bone microenvironment of myeloma patients via single-cell sequencing of trephine bone biopsies.

This study utilised transcriptional data to model intercellular communication between DTCs and the bone microenvironment. Together, these data suggest osteoblastic MSCs are a key component of the dormant tumour cell niche and identify pan-cancer therapeutic targets as candidates to prevent recurrence of skeletal metastases.

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Sequential multi-dose zoledronate therapy prevents rebound bone loss following withdrawal of RANKL inhibition

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Denosumab is an effective osteoporosis treatment, preventing bone loss by inhibiting RANKL. However, stopping denosumab leads to rebound bone mineral density(BMD) loss due to accelerated bone resorption by osteoclasts observed as increasing bone resorption markers. Sequential treatment with zoledronate(ZOL) after denosumab does not consistently prevent this bone loss in patients. We hypothesised that sequential multi-dose ZOL would prevent bone loss following withdrawal of RANKL inhibition.

Ten-week-old female C57BL/6 mice were treated with 2-weeks of thrice-weekly saline(vehicle) or OPG:Fc(10mg/kg) to inhibit RANKL. Mice were then treated with single-dose ZOL(0.01mg/kg) at week 5(OPG+SZ), double-dose ZOL(OPG+DZ) at weeks 5+12, or saline(OPG+S). Longitudinal BMD and serum TRAP5b were measured. MicroCT and histology were performed on samples harvested at week17.

Following OPG:Fc, BMD peaked at week10 in all OPG-treated groups($p<0.0001$ vs vehicle. By week14, BMD normalised to vehicle levels in OPG+S mice. OPG+SZ maintained higher BMD than OPG+S to study end($p<0.01$). Importantly, BMD remained higher in the OPG+DZ group compared to both OPG+S and OPG+SZ groups($p<0.001$), from week10 to study end(A).

MicroCT showed higher femoral trabecular volume(BV/TV) in both OPG+SZ and OPG+DZ groups by 20 and 34% respectively compared to OPG+S and vehicle($p<0.001$)(B). Serum TRAP5b was significantly reduced compared to vehicle following OPG:Fc in all treated groups(C). TRAP5b rose rapidly in OPG+S mice at week 12 to levels 68% higher than vehicle($p<0.0001$). In ZOL-treated mice, TRAP5b rose to reach vehicle levels at week 12 and remained equivalent to vehicle to week17. Histological TRAP analysis showed higher osteoclast numbers in both OPG+SZ and OPG+DZ groups compared to vehicle and OPG+S mice, despite equivalent serum TRAP5b(D).

Our findings show that multiple-dose sequential ZOL is superior in preventing bone loss following withdrawal of RANKL inhibition through suppressing osteoclast function, with no impact on rebound osteoclast formation. A multi-dose sequential zoledronate strategy could be considered following denosumab cessation.

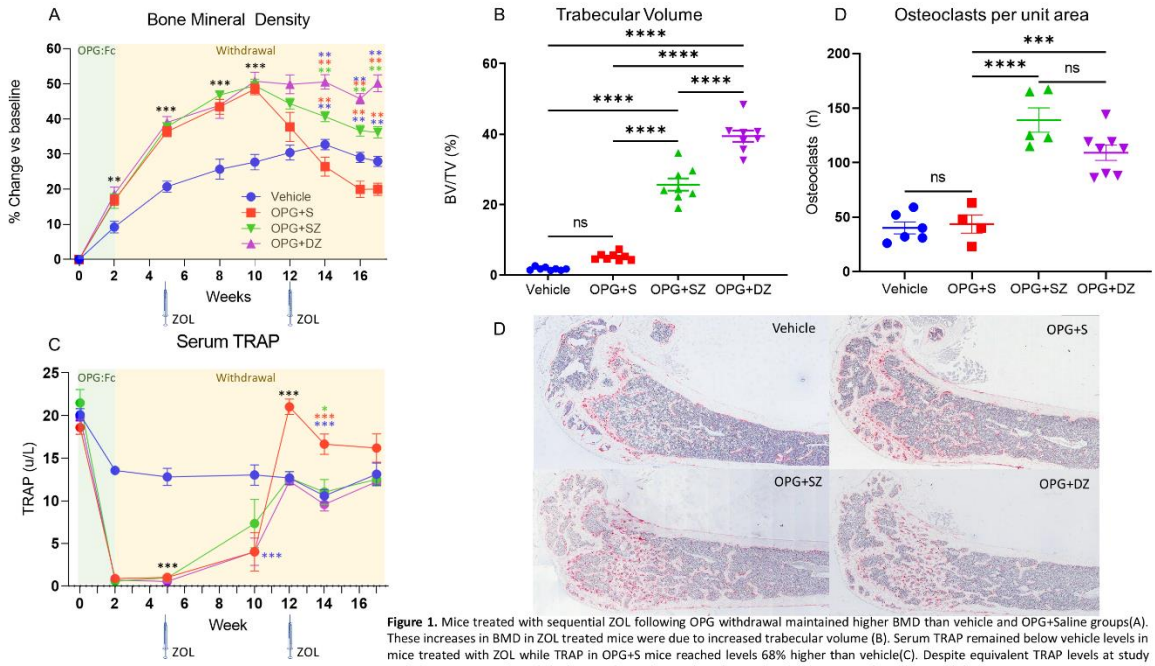

Figure 1. Mice treated with sequential ZOL following OPG withdrawal maintained higher BMD than vehicle and OPG+Saline groups(A). These increases in BMD in ZOL treated mice were due to increased trabecular volume (B). Serum TRAP remained below vehicle levels in mice treated with ZOL while TRAP in OPG+S mice reached levels 68% higher than vehicle(C). Despite equivalent TRAP levels at study end, there were significantly higher numbers of osteoclasts (stained red) in ZOL treated mice femurs (D).

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Self-assessed limited mobility is associated with increased fracture risk in both women and men in the 45 and Up Study

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Measured poor physical performance is associated with increased fracture risk. However, whether self-assessed limited mobility is associated with fracture is unknown.

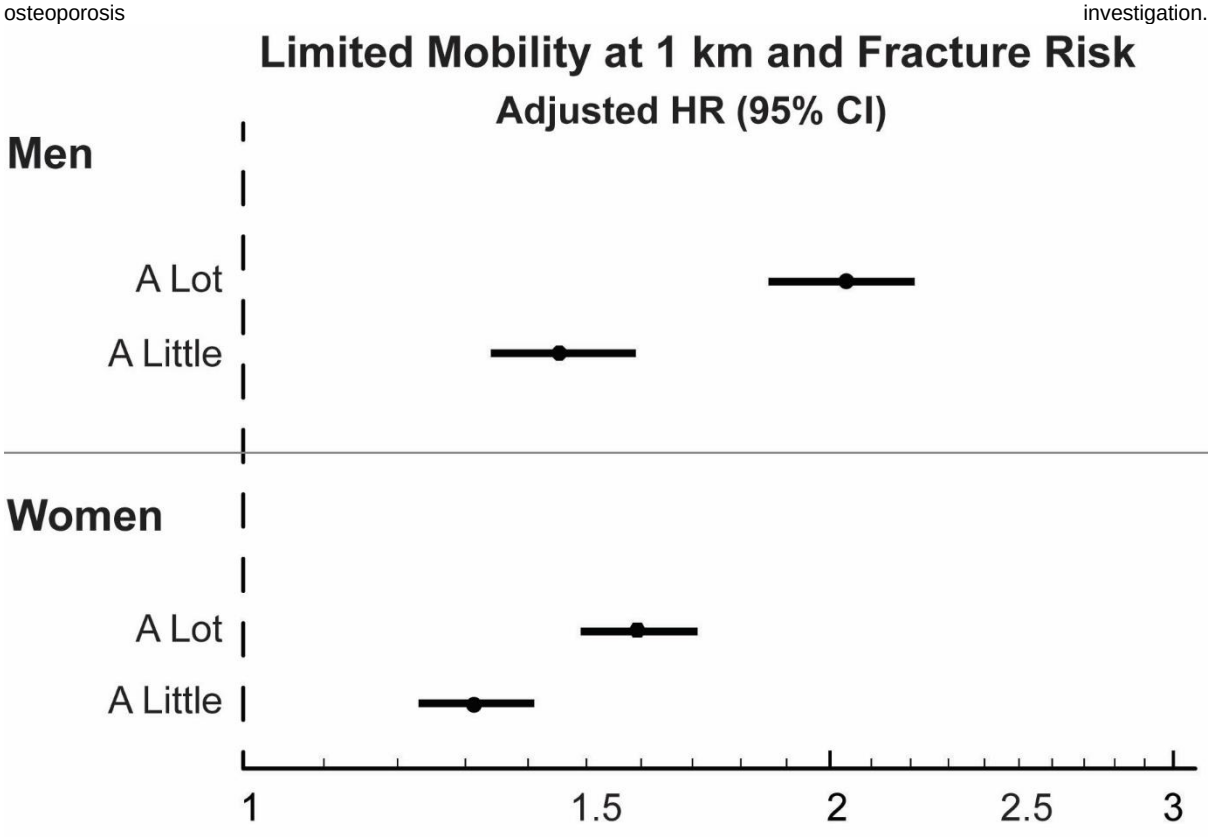
This study aimed to determine the association between self-reported limited mobility and 5-year fracture risk.

45 and Up is a prospective population-based cohort study with questionnaire data linked to mortality and hospital records. A cohort of 122,233 women and 110,365 men with baseline questionnaire data on limited mobility at 1 km self-assessed as “Not at all,” “A little” and “A lot” was selected. Fracture events were ascertained from hospital records. Fracture risk was estimated using gender-specific Cox proportional hazards models adjusted for age, weight, falls, and prior fracture.

Approximately 22% of women and men reported limited mobility (12% - “A little” and 10% - “A lot”) at baseline. During the first 5 years of follow-up, 6867 women and 4155 men experienced a minimal trauma fracture. Individuals with fracture were older, had more comorbidities, falls and prior fractures and were more likely to report limited mobility. After multivariable adjustment, limited mobility was associated with ~32% to >2-fold greater fracture risk [“A little”: 1.32 (1.23 - 1.41), and 1.46 (1.34 - 1.59); “A lot”: 1.59 (1.49 - 1.71) and 2.02 (1.86 - 2.21), for women and men, respectively](Figure). Limited mobility was significantly associated with fracture risk at all sites. The magnitude of association for all degrees of limited mobility was highest for hip (HR 2.16 - 3.34) followed by vertebral (HR 1.56 - 2.21) and non-hip non-vertebral fracture (HR 1.20 - 1.71).

All degrees of self-reported limitation in mobility is associated with increased fracture risk over and above known fracture risk factors. This study suggests that this simple assessment may be a useful clinical tool to select candidates who would benefit from

osteoporosis investigation.



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A global cardiovascular pharmacovigilance study of bisphosphonate and teriparatide users

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Publish consent withheld

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Orphan Receptors Regulate Ovaries and Rule Reproduction

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The orphan nuclear receptors are a subset of the nuclear receptor family for which the ligands have not been defined. We have studied two of these, steroidogenic factor 1 (SF-1, NR5A1) and liver receptor homolog-1 (LRH-1, NR5A2). Both function as transcription factors in the ovary and other tissues and target the same DNA recognition sequence. LRH-1 is specific to the granulosa cells in the ovary, while SF-1 is found in granulosa, theca, and interstitial cells. Conditional depletion of LRH-1 in mice prior to the ovulatory signal via Cre/lox technology obviates ovulation. Global chromatin immunoprecipitation analysis demonstrated extensive reorganization of the LRH-1 cistrome in response the LH surge. After the LH surge, LRH-1 depletion results in impairment of luteal development and function, resulting in infertility. SF-1 depletion before ovulation disrupts follicle development, while depletion after the ovulatory signal compromises both ovulation and luteal function. Recent studies of the ovarian reserve indicate that LRH-1 expression is initiated in a subset of primordial follicles, poising them for entry into the developing pool, an event inhibited by conditional knockout. Downstream effectors include quiescence factors and genes associated with epithelial-mesenchymal transformation of granulosa cells. SF-1 is expressed in the prenatal ovary and depletion beginning prior to birth reduces the assembly of primordial follicles. There is also substantial inhibition of activation of those that develop. Among downstream effectors of SF-1 on the primordial pool are the mTor-Kit signaling system and forkhead transcription factor Foxl2. In spite of employing the same target motif, there seems to be little compensation by either orphan receptor when the other is depleted, either in the ovulatory or perinatal context. In summary, LRH-1 and SF-1 are essential regulators of multiple aspects of ovarian function, from follicle formation, to activation, development, ovulation and luteal function. Supported by CIHR project grant 166020.

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Reproductive tract organoids and their potential applications for improving patient outcomes

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Women represent half of the planet's population, and still, there is limited investment in technologies addressing health conditions affecting women. The majority of clinical testing and trials during the product development stages do not consider women's health and needs. We have very little cellular and molecular understanding of many women's health conditions, partly due to the high morbidity and low mortality associated with these diseases. We have developed organoids from healthy and abnormal human reproductive organs and showed their utility in understanding the complex pathobiology of gynaecological diseases. By applying next generation seq and scRNA seq, we have defined how different subpopulations of cells change during the transition from healthy to disease state. The current work is focussed on utilising these patient-derived models to guide treatments in the clinic.

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Oocyte-secreted GDF9 and BMP15 as serum biomarkers of ovarian function in healthy women and women with PCOS

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Oocyte-secreted growth differentiation factor-9 (GDF9) and bone morphogenetic protein-15 (BMP15) are critical paracrine regulators of female fertility and are predominantly expressed by oocytes. However, it is unknown if serum concentrations reflect changes in ovarian function and/or reproductive endocrine disorders. This study aimed to determine if serum GDF9/BMP15 are associated with ovarian and endocrine parameters, and the ovarian pathology PCOS.

Women aged 21-45 years (n=381) were included from a cross-sectional study at the National University Hospital, Singapore. Participants were volunteers and cases with possible PCOS. Anthropometric measurements, transvaginal ultrasound scans and blood tests were performed, and a questionnaire completed. Serum GDF9 and BMP15 concentrations were determined and analyzed relative to ovarian (cycle regularity, ovarian volume, AFC, AMH), pituitary (FSH, LH, prolactin), estrogenic (estrone, estradiol), androgenic (testosterone, DHT, androstenedione, DHEAS, SHBG, mFG score), and metabolic (BMI, waist-to-hip ratio, insulin, glucose, triglycerides, cholesterol, HDL, LDL, HOMA-IR) characteristics in asymptomatic, PCOM and PCOS women. Statistical analyses used censored regression models and Kendall's tau correlation appropriate for data containing values below the limit of detection.

Serum GDF9 and BMP15 were detectable in 40% and 41% of women, respectively. Serum GDF9 positively correlated with ovarian volume (p=0.02), AFC (p=0.004), and weakly with AMH (p=0.05). Furthermore, irregular menstrual cycles were associated with high GDF9 (p=0.005), and similar, although non-significant associations were seen for BMP15. When stratified into PCOS (n=130), PCOM (n=59), and control (n=192), GDF9 and BMP15 were not different between these groups, and were not associated with the majority of androgenic features of PCOS. However, the relationship between GDF9 and AFC was significantly different between PCOM, PCOS and control women (p=0.02).

These results suggest that serum GDF9 and BMP15 concentrations reflect ovarian but not androgenic characteristics of PCOS, and that the relationships between GDF9 and AFC may be aberrant in women with PCOM/PCOS.

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Inhibin inactivation in female mice leads to elevated follicle stimulating hormone levels, ovarian stimulation and reproductive failure

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Inhibins are heterodimeric members of the transforming growth factor-β (TGF-β) family, composed of a common α-subunit disulfide-linked to one of two β-subunits (βA in inhibin A, or βB in inhibin B). Gonadal-derived inhibin A and B act in an endocrine manner to suppress the synthesis of follicle stimulating hormone (FSH) by pituitary gonadotrope cells. Roles for inhibins beyond the pituitary, however, have proven difficult to delineate because deletion of the inhibin a -subunit gene results in unconstrained expression of activin A and activin B (homodimers of inhibin b-subunits), which triggers gonadal tumorigenesis and lethal cachectic wasting. Here, we generated mice with a single point mutation ($Arg^{233}Ala$) in the inhibin a-subunit that prevents cleavage between the pro- and mature domains. *In vitro*, this mutation blocked inhibin maturation and bioactivity, without perturbing activin production. Characterisation of $Inha^{R233A/R233A}$ mice indicated that FSH levels were elevated 2-3-fold, due to the lack of negative feedback from inhibins, but no pathological increase in activins was observed. While inactivation of inhibin A and B had no discernible effect on male reproduction, female $Inha^{R233A/R233A}$ mice had enlarged ovaries, owing to increased antral follicle and corpora lutea number, and enhanced natural ovulation rates. Fertility analyses revealed that inhibin inactivation resulted in

significant embryo-foetal resorptions and severe subfertility, likely owing to disrupted maternal ovarian function. Intriguingly, heterozygous *Inha*$^{+/R233A}$ mice had enhanced fecundity, carrying twice the number of healthy pups to term during pregnancy, relative to wild-type littermates. These studies have revealed novel roles for inhibins in the establishment and maintenance of pregnancy, and demonstrated that partial inactivation of inhibin A/B is an attractive approach for enhancing female fertility.

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Dapagliflozin restores ovulation in female melanocortin-4-receptor knock-out (MC4R KO) obese mice

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Infertility occurs more significantly in overweight and obese women with disruption of reproductive hormone profiles. Although the clinical impact of obesity on female infertility has been extensively studied with clearly demonstrated improvement by reducing weight, the effective medical treatment is still a distance away due to unclear pathological molecular mechanisms. MC4R KO mice are an obese model with reported hyperphagia, obesity, hyperglycemia, hyperinsulinemia, insulin resistance, and hepatic steatosis. This mouse line was an overeating obese model with remarkable female reproductive hormone disturbance, dysregulated estrous cycle, and significantly reduced formation of corpus luteum (CL)[1]. Dapagliflozin is an SGLT2 inhibitor used clinically in the treatment of diabetes with the demonstrated improvement of obesity in this mouse model[2]. In this experiment, dapagliflozin treatment (1 mg/kg/day for 14 weeks from 14-week-old) of MC4R KO female mice improved glucose tolerance, restored partially the pulsatile profiles of growth hormone (GH) and luteinizing hormone (LH), including the amount of pulsatile secretion, mean pulse mass, and pulsatile secretion, and changed approximate entropy and secretion mode. The estrous cycle was partially and significantly normalized, and the number of CL was markedly increased in female MC4R KO mice by the dapagliflozin treatment. The expression of genes related to reproductive regulatory factors and hormones in the hypothalamic and pituitary was significantly elevated by the dapagliflozin treatment. Based on the above data, it may be concluded that dapagliflozin recovers reproductive function in an obese mouse model through recovering reproductive endocrine profiles. Dapagliflozin treatment may potentially be useful in the treatment of infertility in clinic obese patients.

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Inflammation mediates the effects of peri-pregnancy diet on the maternal brain

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Maternal mood disorders are serious complications of pregnancy experienced by as many as 1 in 5 mothers worldwide. Maternal obesity increases the risk of postpartum mood disorders, but the mechanisms are unknown. Here we examined the effects of maternal obesity, induced by the consumption of a high-fat-high-sugar (HFSD) diet before and during pregnancy on postpartum brain and behaviour in rats. We also assessed if the effects of HFSD could be reversed by consumption of a healthier diet during pregnancy, specifically by a diet replete in omega-3 polyunsaturated fatty acids. Our data show that consumption of HFSD before and during pregnancy activated magnocellular, but not parvocellular, neurons in the paraventricular region of the hypothalamus (the apex of the stress axis), and only moderately affected anxiety-like behaviours. However, HFSD-induced pre-conception obesity was associated with elevated levels of inflammatory cytokines and reduced microglial complexity; morphology indicative of microglial activation. A shift to a healthier diet during pregnancy reversed systemic and neuro-inflammation. Surprisingly, both HFSD and omega-3-replete diet increased the numbers of newborn neurons in the hippocampus. While outside of pregnancy neurogenesis refines hippocampal activity, the opposite occurs postpartum, where increased neurogenesis may facilitate mood disorders. These data highlight the potential role of inflammation in mediating the effects of diet on the maternal brain and support the importance of a balanced dietary intake before and during pregnancy. Our findings also indicate the need for future research into key triggers that may influence the neuroimmune balance in the maternal brain.

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Gender affirming hormone therapy induces specific DNA methylation changes in blood

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DNA methylation is an epigenetic mark capable of modulating gene expression, and is influenced by a combination of genetics, environmental factors, and aging. Sex-specific methylation patterns are widespread across autosomal chromosomes and can be present from birth (i.e. sex-specific but *not* age-related) or arise over the lifespan (i.e. sex-specific *and* age-related). In individuals where gender identity and sex assigned at birth are incongruent, as in the case of transgender people, feminization or masculinization may be sought through gender affirming hormone therapy (GAHT). Previous studies have shown that periods of hormonal change, including puberty, pregnancy, and menopause, can affect blood methylation patterns, but GAHT-induced changes have not yet been fully characterized. We profiled genome-wide DNA methylation in blood of transgender women and transgender men prior to and after 6 and 12 months of GAHT. We identified several thousand differentially methylated CpG sites (DMPs) and several differentially methylated regions (DMRs) across both feminizing and masculinizing hormone therapy, with the majority showing progressive changes across GAHT. Genes with GAHT-associated DMPs in promoter regions were enriched for numerous immune and endocrine signaling processes. We found that sex-specific DNA methylation patterns already established at birth are largely refractory to GAHT-associated changes. GAHT did, however, alter a small proportion of sex-specific CpGs, with enrichment for age-related changes – and more specifically – adolescence-associated changes. Importantly, sex-specific CpGs altered by GAHT showed a consistent shift towards the methylation pattern of the GAHT-naïve opposite sex. These results provide novel insight into the effects of GAHT on the blood methylome and add perspective to the complex interplay of sex hormones, sex chromosomes, and DNA methylation in the context of immunity.

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Exploring a potential genetic basis of gender identity

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Publish consent withheld

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Coproduction is key

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Experience as an academic and as an academic leader

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Experience with Industry

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BAM15: an innovative therapeutic approach to manage PCOS metabolic features associated with PCOS?

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Polycystic ovary syndrome (PCOS) is a prevalent heterogeneous disorder characterized by endocrine, reproductive and metabolic abnormalities. To date, there is no cure for PCOS, and existing treatments are suboptimal. Obesity and adverse metabolic features are prevalent in PCOS patients, but weight loss has a favorable effect on PCOS features. However, dietary interventions aimed at weight loss are difficult to maintain long-term. Interestingly, recent data from animal studies has shown that a small molecule mitochondrial uncoupler, BAM15, is an effective approach to pharmacologically treat obesity and metabolic diseases. Therefore, this study aimed to investigate the efficacy of BAM15 to improve PCOS-traits in a hyperandrogenic PCOS mouse model. As expected, exposure of female mice to dihydrotestosterone (DHT) induced the PCOS metabolic features of increased body weight ($P<0.05$), lean mass ($P<0.001$), increased parametrial and mesenteric fat pad weights ($P<0.05$) and adipocyte hypertrophy ($P<0.05$). DHT-induced PCOS mice also exhibited increased HOMA-IR, cholesterol and fasting triglyceride levels and hepatic steatosis (all $P<0.05$). Conversely, DHT-induced PCOS females treated with BAM15 displayed lowered body weights similar to controls, a significant decrease in parametrial and mesenteric fat depot weights ($P<0.05$) and a slight decrease of adipocyte hypertrophy. Likewise, BAM15 treatment moderately decreased HOMA-IR, cholesterol and fasting triglyceride levels and the degree of hepatic steatosis observed in PCOS females, to levels comparable with controls. Furthermore, PCOS mice displayed the reproductive PCOS features of irregular cycles and anovulation, which were not ameliorated by BAM15 treatment. These findings demonstrate that the pharmacologic mitochondrial uncoupler BAM15 ameliorated metabolic PCOS features in a preclinical mouse model of PCOS. Altogether, these data provide evidence to support BAM15 as a possible innovative therapeutic approach to manage PCOS associated metabolic features.

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Polycystic Ovary Syndrome; Insulin Resistance and Data From Cohort Studies

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Polycystic Ovary Syndrome (PCOS) affects 8 to 13% of Australian reproductive-aged women and is a major public health concern. Whilst reproductive features (anovulation, infertility) are prominent, PCOS also has major metabolic [obesity, metabolic syndrome, type 2 diabetes (T2DM), cardiovascular disease risk factors] and psychological features.

Obesity is a major chronic disease, with rising prevalence and diverse health impacts. The interplay between PCOS and weight contributes to the long-term consequences of PCOS, but is not well understood. Women with PCOS demonstrate insulin resistance, which leads to adverse health consequences, both independent of and exacerbated by obesity. Women with PCOS were more insulin resistant than body mass index (BMI)-matched controls on euglycaemic hyperinsulinaemic clamp studies. Insulin resistance was present in 75% of lean women with PCOS, 62% of overweight controls and 95% of overweight women with PCOS.

There is a lack of community-based studies exploring the natural history of polycystic ovary syndrome (PCOS). The Australian Longitudinal Study on Women's Health (ALSWH) is a large community-based prospective study, which has collected data from approximately 9000 reproductive-aged women at seven time points over 19 years. The Raine cohort from Western Australia included 2868 pregnant women with ongoing long-term family follow-up. It is one of the largest successful prospective cohorts of childhood, adolescence, and pregnancy in the world, with 80% of participants still active and 11 time points over 26 years with clinical data, bloods and imaging. Reproductive, metabolic and psychological outcomes will be presented from both cross-sectional and longitudinal analysis of the above datasets.

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Androgen Use, Misuse and Abuse

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Content coming soon

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Steroid Metabolomics for the Exploration of Adrenal Disease

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Available Soon

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Reproduction and Development of the Short-Beaked Echidna

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The short-beaked echidna (*Tachyglossus aculeatus*) is one of only four extant species of egg-laying mammals (Monotremata: three echidnas and one platypus), but despite its common and ubiquitous distribution throughout Australia, information on its

reproductive biology is limited. What is known is that the reproductive biology of the echidna is anatomically and behaviourally distinct in comparison with both marsupial and eutherian mammals. After a short gestation, the egg is incubated in the pouch and once hatched, the young puggle continues its development while residing in the pouch, sucking milk form the mammary patches.

In conjunction with Currumbin Wildlife Sanctuary (Queensland) we have been investigating echidna reproduction and development in detail for the first time. The newly hatched pouch young is developmentally remarkably similar to a marsupial neonate, but has a number of distinct differences, particularly with regards to their sexual differentiation. The developing gonads have a typically mammalian embryonic appearance, but their differentiation is delayed compared to marsupials. Together with the platypus, the echidna has a unique sex chromosome make-up (echidna 5Xs:4Ys; platypus 5Xs:5Ys), but neither have the sex determining gene *SRY*. However, many of the other common mammalian sexual differentiation genes are present in the developing echidna gonads. The adult male echidna has a unique phallus with four glans penises, but at ejaculation only two of these become erect. This is due to the presence of a split urethra and two separate corpora spongiosa for the entirety of its length. Whilst the phallus is present at hatching as a distinct protuberant structure in the young, urethra patency is delayed since they excrete both gut contents and urine from a single hole: the cloaca. Together, these results provide the first insights into the evolution of the sexual development pathways of the monotremes and how they differ from the marsupials and eutherians.

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The Genomic Basis of Sex Change in Fish

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Fishes exhibit remarkably diverse and plastic sexual development. This includes functional adult sex change, which has evolved repeatedly across the teleost tree of life. While the sex change process and its evolutionary advantages are well known, how environmental cues drive such dramatic changes in sexual identity, and the molecular processes involved, have been longstanding questions. Female-male sex change (protogyny) is common in the wrasses (Labridae), where individuals reproduce first as females, but routinely reverse sex in the absence of a socially dominant male. This process involves complete gonadal restructuring where no identifiable male tissues exist in the gonad prior to sex change. Using social manipulations to generate a sex change time-series in each of two distantly-related wrasses - the Caribbean bluehead (Thalassoma bifasciatum) and New Zealand spotty (Notolabrus celiodotus) – together with transcriptomic and whole-methylome approaches, Erica and colleagues have zeroed in on the primary trigger and subsequent molecular cascade that transforms female into male. In this talk, Erica discusses the extensive transcriptional and epigenetic reprogramming that occurs in gonadal cells during sex change, and highlights both conserved and derived aspects of the molecular network that orchestrates sex change in these two species. Neofunctionalisation of duplicated sex-pathway genes, epigenetic reprogramming and the stress axis appear to be key molecular components of sex change, yet the proximal molecular trigger that initiates the ovary-testis transformation may be an important point of diversification between species. Understanding the genomic basis of sex change in fish has important implications for understanding the evolution and functioning of vertebrate sex determination and developmental systems more broadly.

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Exploiting the physiological roles of seminal plasma in sheep

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While exposure of spermatozoa to seminal plasma is not an absolute requirement for their subsequent fertility, a growing body of literature has demonstrated the highly varied role of this fluid in reproduction. Our studies have focused on understanding the physiological roles of seminal plasma in sheep, with the ultimate goal of utilising seminal plasma to improve outcomes of artificial insemination with cryopreserved semen. Our insemination trials demonstrated that while seminal plasma does not alter basic semen parameters, it is a key component driving successful cervical transit of ram spermatozoa. Employing proteomics, we showed that seminal plasma and its extracellular vesicles deliver a limited protein cargo to ram spermatozoa, rather than introducing a wide variety of novel proteins. Using flow cytometry and in-vitro binding assays, we also demonstrated that seminal plasma exposure alters the sperm glycocalyx and interferes with sperm-neutrophil binding, potentially contributing to sperm survival within the female tract. Based on our proteomic findings, we subsequently focused on the highly abundant seminal plasma Binder of Sperm Proteins, exploring their physiological roles in ram sperm capacitation, including cholesterol efflux, and their potential application in minimising sub-lethal sperm freezing damage. Further work will focus on using a combination of complementary, physiologically relevant seminal plasma proteins to enhance the field fertility of cryopreserved ram semen.

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Meiotic Executioners and Persistent Y Chromosomes

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The Y chromosome has been touted as a wimpy relic of the X, with its survival dependent on a few critical functions in spermatogenesis and sex determination, the loss of which would signal its demise. Why then has it survived since its origin (~165 MYA) in all but a handful of therian mammal species? This is in stark contrast to the high turnover of sex chromosomes observed in other vertebrate linages, so the mammal Y turns out to be an exception of persistence, rather than the rule. Here we propose a novel explanation for such perseverance. The Y chromosome bears so-called 'executioner genes' that are critical for successful meiotic progression. Their expression is required to initiate meiotic sex chromosome inactivation, but then must be subject to this very silencing they induce to ensure germ cell survival. When executioners are translocated to an autosome they escape meiotic silencing and, being pachytene-lethal, cease meiosis. Therefore, these meiotic executioners act as their own judge, jury and executioner, posing strong evolutionary constraint for the Y chromosome to persist in eutherian mammals.

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IL-1 receptor antagonist rytvela protects against Group B Streptococcus (GBS)-induced preterm birth and fetal loss in mice.

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5. Division of Obstetrics and Gynaecology, The University of Western Australia , Subiaco, WA, Australia

Premature birth is a common and critical health issue in fetal-maternal medicine with long-term consequences especially for early preterm neonates. The pathophysiology is poorly understood and the causal factors often uncertain, but inflammatory mechanisms are clearly implicated. Toll-like receptors (TLRs) are critical upstream gate-keepers controlling the inflammatory activation that precedes preterm delivery and pro-inflammatory cytokine interleukin-1 beta (IL-1b) has been identified as a major upstream product following the activation of the TLR pathway. Previously we have shown that inhibition of IL-1 signaling using rytvela, a non-competitive allosteric peptide inhibitor of IL-1 receptor (IL-1R) signaling, can prevent preterm birth caused by the TLR4 ligand lipopolysaccharide in mice. Here we evaluate the efficacy of rytvela in preterm birth elicited by Group B Streptococcus (GBS), a gram-positive bacteria commonly associated with spontaneous preterm birth in women, that activates inflammation via TLR2 and TLR8. We investigated (1) whether inhibition of IL-1 signalling using rytvela may prevent the parturition cascade caused by GBS-induced inflammation and (2) the consequences of in utero exposure to rytvela for resulting progeny. Pregnant C57Bl/6 mice (n=8-16 dams per group) were administered intrauterine heat-killed GBS ($5x10^9$ IU/100 µl) or PBS, with or without co-administration of rytvela (ip), on gestational day (GD) 16.5 and allowed to progress to birth. Rytvela treatment acted to reduce the rate of GBS-induced preterm delivery from 62% (9/16) to 12% (1/8)($p<0.05$). Viable litter size at birth was increased from 2.7±0.8 to 5.4±0.7 pups per litter ($p<0.05$), and postnatal survival at 1 week was increased from 44% to 62%. These results demonstrate that intervention with rytvela to suppress the IL-1-induced inflammatory cascade can mitigate GBS-induced preterm birth and perinatal death. The data support continued investigation of the IL-1 pathway as a potential target for new prevention or treatment options in women at risk of preterm delivery.

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The (pro)renin receptor regulates amnion matrix degradation and thus may play a role in preterm birth

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Preterm birth (PTB) is the single largest cause of death in infants and young children. 25-30% are associated with preterm premature rupture of membranes (P-PROM). P-PROM is more prevalent in 'male' pregnancies, suggesting that the integrity of the male amnion is less than female amnion. In the kidneys, the (pro)renin receptor ((P)RR) regulates extracellular matrix production by increasing the MMP9:TIMP1 ratio. As expression of the (P)RR is higher in female amnion[1], we postulated that (P)RR in amnion epithelial cells (AECs), like the kidney, regulates membrane integrity in a sex-dependent manner.

To investigate the relationship between (P)RR and membrane integrity, primary human AECs were isolated and transfected with 10 nM (P)RR or scrambled siRNA (n=8/group). Following qRT-PCR validation for (P)RR knockdown, expression of markers of membrane integrity (MMP9 and TIMP1) were determined. MMP activity was also measured using zymography.

In female AECs, MMP9 mRNA expression ($P=0.02$) and metalloproteinase activity ($P=0.003$) was significantly lower than in male AECs. Fetal sex did not affect the expression of (P)RR or TIMP1 mRNA. (P)RR siRNA significantly reduced (P)RR mRNA expression by ~80% in both male and female AECs (both $P<0.0001$). (P)RR siRNA also reduced the expression of TIMP1 mRNA in both male and female AECs ($P=0.006$ and 0.03, respectively) but metalloproteinase activity was only enhanced in female AECs ($P=0.03$).

Overall, female AECs had lower levels of markers of matrix breakdown compared with male AECs. This could account for the lower prevalence of P-PROM in female pregnancies. This sex difference however does not appear to be driven by (P)RR. Inhibition of (P)RR was associated with decreased expression the membrane integrity marker (TIMP1). In female AECs there was also a rise in MMP activity. Thus, the (P)RR is likely to be involved in maintaining amnion integrity and decreased expression of (P)RR might be associated with PTB.

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A novel *in silico* framework of oxygen diffusion reveals reduced placental oxygenation in a rat model of growth restriction

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In silico tools are emerging as a powerful means to overcome current experimental challenges and have the potential to elucidate the subtle, yet convoluted harmonization of vascular structure, haemodynamic, and exchange adaptations of the placenta. We present a computational fluid dynamics model of flow and diffusion, calibrated with a range of bio-fabricated three-dimensional (3D) networks (Fig 1A) and validated with microfluidic flow experiments. We applied established blood flow simulations methods to 3D geometries of rat feto-placental arterial casts, imaged with microCT, obtained from a model of growth restriction (chronic glucocorticoid exposure)(n = 3) and control (n = 3) pregnancies. A computational diffusion model was applied to idealised capillary models coupled to the outlets of the imaged vasculature (Fig 1B) to compute oxygen gain by each terminal vessel of the placenta. Modelling outcomes were validated against magnetic resonance imaging (MRI) of placental oxygenation (ΔT2*) in the same rat model. Capillary velocities were 47% higher in growth restricted placentas compared to controls and as a result, oxygenation was reduced by 74% in growth-restricted models (Fig 1C). Placental ΔT2* MRI imaging (Fig 1D) showed that responses were 70% lower in growth-restricted placentas, in good agreement with computer simulations (<10% error). Here, we show an *in silico* approach that elucidates the mechanisms of impairment of placental function in a rat model of growth restriction. Importantly, modelling of oxygen diffusion accurately recapitulates real-time MRI assessments, highlighting the potential of *in silico* approaches to predict and diagnose placental dysfunction.

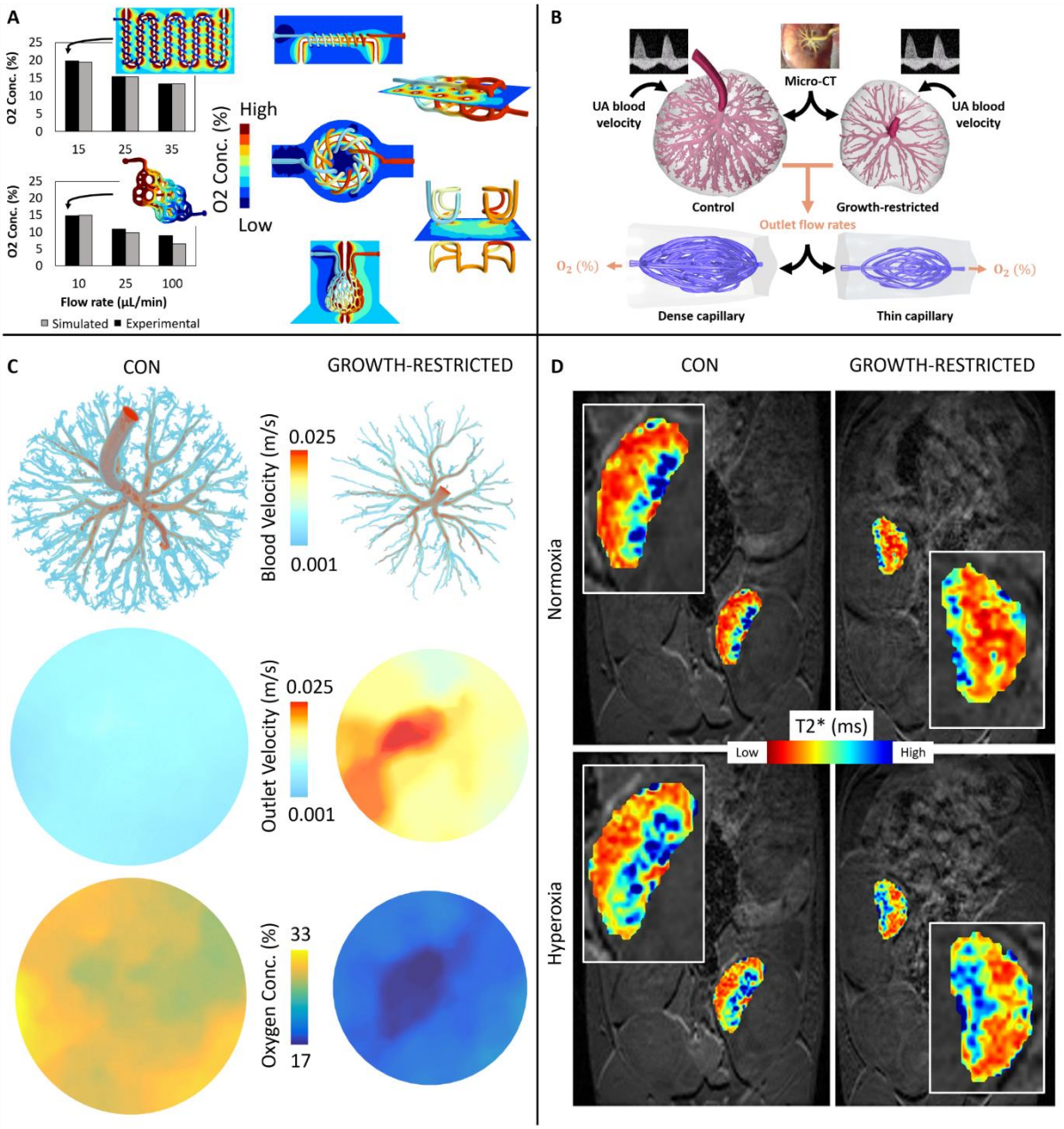

Figure 1: *in silico* framework. Open-source bio-mimetric networks used for method development (A) before application to feto-placental arteries, coupled with idealised capillaries (B). Simulations reveal a gradual deceleration of blood and projections of outlet velocities and oxygenation show impairment in growth restriction (C). Representative 2D slices for normoxia and hyperoxia from MRI T2* images stacks (D).

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The transgenerational effect of diethylstilbesterol on female fertility

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Significant decreases in both male and female fertility have been observed over the past 50 years, with female conceptions rates dropping by 44% and male sperm counts decreasing by over 50%. This dramatic decrease in fertility can be attributed in part to our increasing exposure to endocrine disrupting chemicals (EDC's). Diethylstilbesterol is an estrogenic EDC that was prescribed to millions of pregnant women between 1940-1970 and resulted in detrimental reproductive effects in the offspring that were exposed *in utero*. Women who were exposed to DES *in utero* experienced higher rates of infertility, pregnancy complications and reproductive cancers. Alarmingly, there is evidence to suggest that these effects may persist in the grandchildren and great grandchildren of exposed women. To determine if exposure to DES can result in transgenerational impacts mice were exposed to 100ug/kg of DES every second day from days 9-17 of gestation as this period of fetal sexual differentiation and development is particularly vulnerable to EDCs. The effects of DES were monitored in the F1-F4 female descendants. Pregnancy rates for F1, F2 and F3 DES females were reduced compared the controls and the fertility index also decreased in the F1, F2 and F3

generations. The onset of puberty was also affected in these females, with the vagina opening significantly earlier in the F1, F2 and F4 females compared with controls. These results indicate a transgenerational effect of DES on fertility and timing of puberty. This has implications for the 50 million DES descendants as well as raising health concerns for the ongoing health impacts caused by exposures to other estrogenic EDCs which are pervasive in our environment.

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Maternal late gestation undernutrition does not affect fetal hepatic or placental cytochrome P450 activity

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Background: Alterations to the intrauterine environment, such as those brought on by maternal undernutrition, can disrupt fetal growth and development and consequently perturb certain physiological processes within the feto-placental unit, including feto-placental metabolism. It is not known how perturbations such as hypoglycemia alters the capacity to appropriately metabolise exogenous chemicals including drugs and other xenobiotics, which are taken in most pregnancies. Drug metabolism is mediated by cytochrome P450 (CYP) drug metabolising enzymes in the . Hypoglycemia reduces activity of CYP isoenzymes, reducing efficacy and safety of certain pharmaceutical therapies. We therefore hypothesised that in a sheep model of late gestation undernutrition (LGUN) feto-placental CYP activity would be reduced, and that fetal glucose infusion (LGUN+G) would rescue reduced CYP activity.

Methods: At 115d gestation (term, 150d), ewes were allocated to control (100% metabolic energy requirement (MER); n=11), LGUN (50% MER; n=7) or LGUN+G (50% MER + glucose infusion via fetal femoral vein; n=6) and maintained on the diet until post-mortem. Microsomes were isolated from placenta and fetal liver collected at 139-142d gestation. Placental and hepatic microsomes were incubated with specific CYP probes and the concentration of metabolite produced was measured using Liquid Chromatography – tandem mass spectrometry (LC-MS/MS).

Results: CYP3A was not detectable in either placenta or fetal liver, and CYP1A2 was not detectable in the fetal liver. Placental-specific CYP1A2 and CYP2D6 activity and hepatic-specific CYP2D6 activity were not affected by LGUN or LGUN+G. CYP2D6 activity was significantly higher in the liver than placenta, irrespective of study group.

Conclusions: The physiological response to LGUN does not affect placental- or hepatic-specific activity of certain CYPs. Our study is the first to report functional activity of CYP1A2 and CYP2D6 in the sheep placenta and provides an experimental workflow to examine the activity of other CYP isoenzymes in the feto-placental unit.

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A novel multicellular 3D model of early pregnancy placental tissue

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Introduction: Preeclampsia affects 5-8% of pregnancies and is the leading cause of maternal and foetal death in pregnancy. Despite this, there are still no effective prevention or treatment strategies. The root cause of preeclampsia is placental dysfunction, however, research into the mechanisms of pathogenesis has been impeded by a lack of reliable models of the human disease. Inadequate trophoblast remodelling of maternal uterine arteries is a major contributing factor in the development of preeclampsia. Thus, a low-cost and reproducible 3D migration and invasion trophoblast model would be transformative for future research and could lead to better management of preeclampsia.

Aim: To establish a novel 3D cell model of the early placenta using a custom-made first trimester trophoblast cell line.

Methods/Results: Organoids were generated by manual seeding of trophoblasts in Matrigel or bioprinting using a RASTRUM 3D cell culture platform and cultured with normal growth medium. Live cell imaging revealed spontaneous organoid formation from single cells within a few days. Trophoblast organoids demonstrated invasive capabilities within the matrix and the emergence of single cells. For histological analysis, a subset of organoids were harvested and processed for haematoxylin and eosin staining. To evaluate the differentiated cell morphologies within the organoids, another subset of organoids were fixed *in situ* and probed with immunofluorescence antibodies against subtype-specific markers including E-cadherin for villous trophoblasts and human leukocyte antigen G (HLA-G) for extravillous trophoblasts.

Conclusions: Our novel 3D trophoblast organoid model recapitulates key trophoblast subtypes of early placental tissue. In particular, the spontaneous differentiation of trophoblast subtypes within these organoids presents an opportunity to investigate key mechanisms involved in trophoblast proliferation, differentiation and cellular function important for placental development. Further, this 3D first trimester trophoblast model could lend significant insight into the key features of placental dysfunction, such as that seen in infertility, miscarriage and preeclampsia.

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Proinflammatory phenotypic plasticity of maternal regulatory T cells precedes interleukin 1β-induced preterm birth in mice

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Maternal immune system dysfunction, notably deficient CD4+Foxp3+ regulatory T (Treg) cells, are implicated in the pathophysiology of preterm birth (PTB). During parturition there is a relative shift away from immune tolerance towards proinflammatory T cell responses. However, the role of Treg cells in parturition is ill-defined. Given that Treg cells exhibit plasticity and adopt T effector-like functions in inflammatory environments, we hypothesised that preterm parturition may be exacerbated by maternal Treg cells with an aberrant inflammatory phenotype or reduced lineage stability. To investigate this, we characterised Treg cells in physiological parturition and sterile inflammation-induced PTB. Female C57Bl/6 mice mated to BALB/c males were administered 6µg interleukin 1β (IL1β) (or vehicle, n=11 mice/group) on gestational day (GD) 15.5 to induce PTB. T cells in the uterus-draining lymph nodes were assessed by flow cytometry on GD16.5, approximately four hours prior to preterm delivery. Mice administered IL1β had expanded T cell populations prior to labour (P<0.010). Treg cell-specific upregulation of activation maker CD25 (P<0.001) and an increased ratio of Treg cells to proinflammatory IFNγ+ T effector cells (P<0.050) suggested an activated Treg cell population may constrain inflammation ahead of PTB. However, there was a subset of Treg cells expressing heightened levels of inflammatory trafficking receptor CCR6 and proinflammatory cytokine IL17A (P<0.050). Using a mouse model that reports Foxp3+ Treg cell lineage, we showed that the number of Treg and so-called "exTreg" cells that had lost expression of Foxp3 was unchanged over GD16.5-18.5, suggesting stability of Treg cells is maintained in normal late gestation. Interestingly, decreased CD4+ T cell expression of master regulator Tbet was evident in physiological late gestation in the lineage tracing mice (P<0.010), and in the PTB cohort (P<0.050). Ongoing studies will investigate the lineage stability of Treg cells prior to PTB, and the significance of modulated Tbet expression during parturition.

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IL11 activates the placental inflammasome, causing pyroptosis and fibrosis and leading to preeclampsia.

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Preeclampsia is a life-threatening disorder of pregnancy unique to humans, diagnosed by sudden onset hypertension (>20 weeks gestation) plus one other complication. Abnormal placental inflammasome activation is associated with preeclampsia, however the cause of excess placental inflammasome activity is unknown. Interleukin (IL)11 is elevated in 1st trimester maternal serum of pregnancies that subsequently develop early-onset preeclampsia. IL11 administration to pregnant mice recapitulates preeclampsia-like features (hypertension and proteinuria). We hypothesized IL11 activates placental inflammasomes to cause preeclampsia.

Pregnant female C57BL6J wild-type mice and mice which lack the inflammasome adaptor component ASC (ASC-/-) were subcutaneously injected with PEGylated (PEG)IL11 (500µg/kg/day) from embryonic day (E)10-16 (n=4-6/group) and systolic blood pressure (sBP), proteinuria, serum sFlt-1, placental formation, fetal/pup growth and placental/renal inflammasome activation measured. Human 1st trimester placental villous explants (n=6-9/group) were treated with IL11 (100ng/ml) +/- MCC950 (5µM, NLRP3 inflammasome inhibitor) for 72h and inflammasome activation measured.

PEGIL11 treatment caused preeclampsia-like features (hypertension, proteinuria), activated placental and renal inflammasomes (cleaved caspase-1, IL1β, gasdermin-D) and caused placental and renal fibrosis only in wild-type mice: no effect was seen in ASC-/- mice. However, preventing PEGIL11-induced placental inflammasome activation did not ameliorate PEGIL11-induced placental damage: PEGIL11 treatment impaired labyrinth trophoblast differentiation, elevated circulating sFlt-1 and caused fetal growth restriction and stillbirth in both wild-type and ASC-/- mice. In human placental villous, IL11 activated cytotrophoblast inflammasomes (caspase-1 cleavage, IL1β secretion) resulting in cytotrophoblast pyroptosis (cleaved gasdermin-D and LDH release), which was inhibited by MCC950 co-treatment.

For the first time we demonstrated that IL11 activates the inflammasome, causing trophoblast pyroptosis and placental and renal fibrosis. Inhibition of placental/renal inflammasome activation prevented the maternal symptoms of preeclampsia. However, as IL11-induced placental damage was not ameliorated by loss of ASC, inhibition of inflammasome activity in the placenta without inhibition of other IL11 signaling pathways may not prevent adverse fetal outcomes.

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Dysregulation of the Male Gonadal Axis due to Energy Deficit

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While gonadal axis dysregulation due to energy deficit is well recognised in women, the effects of energy deficit on the male gonadal axis have received much less attention. In this presentation I will cover the effects of energy deficits (both absolute and relative) on the male gonadal axis, highlighting the role of excessive exercise (i.e., Relative Energy Deficit of Spoert (RED-S), from a clinical and mechanistic perspective, provide recommendations for management, and highlight knowledge gaps and opportunities for future research.

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Optimizing Performance in Female Athletes: A Spotlight on Hormonal Contraception

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This presentation will provide an overview of the determinants of female athletic performance including the contribution of participation, injury prevention, program prescription and training environment, as well as talent identification, and profile. A focus on sports science & sports medicine will explore current trends in female athlete research. Particular attention will be given to the interaction of hormonal contraception with performance and provide current knowledge and remaining questions in this area of research. Details of the Australian Institute of Sport's Female Performance & Health Initiative will be presented and details of the vision, mission, and three pillars of focus will be explored.

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Exercise and bone turnover markers: implications for health

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The skeleton has protective, mechanical and metabolic roles. Bone should be strong to prevent fractures, but light, enabling movement in a gravitational environment. Bone remodelling, the cellular machinery responsible for bone integrity and strength, is a finely balanced process responsive to mechanical loads and hormonal changes. As bone remodelling requires energy, it was hypothesised that hormones produced by bone may have endocrine-like effects beyond the skeleton. Indeed, in recent years it was discovered that a crosstalk exists between bone and skeletal muscle.

Exercise is a non-pharmacological intervention that improves bone and muscle health simultaneously, reducing the risk for osteoporosis and sarcopenia, as well as improves glucose disposal, reducing the risk of type 2 diabetes. As such, exercise can be used as a tool to uncover the interaction/s between bone and muscle, as well as mechanisms involved in this crosstalk. Indeed, it was reported that the circulating levels of some bone hormones (such as osteocalcin and osteoglycin) are not only related/correlated with insulin sensitivity and improved glucose control, but also have a direct effect on these measures.

This presentation will explore the effects of exercise on BRMs as well the link between BRMs, in particular osteocalcin, and skeletal muscle function and metabolism at rest and post-exercise. A better understanding of the mechanisms behind the interactions of exercise, BRMs and glucose regulation may provide new pharmacological and non-pharmacological avenues to prevent and manage diseases such as type 2 diabetes and sarcopenia.

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Detection of Designer Steroids Using Androgen Bioassays

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Androgens remain the most widely abused prohibited substances in sports. Presently, detection of androgen abuse in sports relies on using gas chromatography-tandem mass spectrometry (GC-MS/MS)-based techniques. While exquisitely sensitive, these techniques are only as good as their reference libraries. If the library does not contain reference material for the targeted compound, the GC-MS/MS approach can fail. The failure of the GC-MS/MS approach is most apparent when designer androgens with novel structures are present and bypass targeted detection. Designer steroids are marketed as sports supplements, often with their true identity masked. Given that these novel structures need to be captured if anti-doping testing is going to be meaningful, a non-targeted catch-all approach for androgens is required. Androgen receptor-based bioassays represent a non-targeted approach that exploit the common androgen signaling pathway. The bioassays mimic biology whereby androgens activate AR, triggering the translocation of AR into the nucleus where AR binds to androgen response elements (ARE) in the genome to enhance expression of androgen response genes. Commonly, yeast- or mammalian cells are genetically modified to overexpress AR and to harbor a synthetic DNA transcript that encodes an ARE upstream of a reporter gene, which when expressed acts as a biosensor for androgens. Cell-based bioassays have been used to identify presence of designer steroids in athlete's biological samples, as well as sports supplements. Cell-based bioassay execution is, however, challenging and can be both labor intensive and time consuming. For these reasons, cell-based bioassays are unlikely to be used as a screening approach for anti-doping laboratories despite their proven non-targeted approach. To overcome the complexity of cell-based androgen bioassays, cell-free androgen bioassays have recently emerged as a high-throughput screening option. Application of this new state-of-the-art methodology, complementary to the targeted screening approaches, will increase the chance of detecting designer androgens.

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SPINT2 expression in pregnancies complicated by placental insufficiency

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Poor placental implantation can lead to preeclampsia and/or delivery of a small for gestational age (SGA) fetus. Diagnostic tools to predict placental insufficiency have limited accuracy. We recently reported circulating SPINT1[1] to have the strongest association with placental insufficiency of any protein. SPINT1 is a protease inhibitor expressed on the surface of trophoblasts and secreted into the maternal circulation. Here we sought to investigate SPINT2, a structurally and functionally related protein of SPINT1.

We measured circulating SPINT2 in several large cohorts: 1) a prospective case-cohort collection at 36 weeks' gestation (n=326, cohort 1 from Australia); 2) prospective 24-34 weeks' gestation samples (n=132, cohort 2 from UK); and, 3) patients with established preterm preeclampsia (cohort 3, Australia). Plasma SPINT2 was elevated prior to diagnosis at 36 weeks' gestation in women who later developed preeclampsia (p=0.028; cohort 1) or delivered an SGA baby (p=0.002, cohort 1). However, no change was observed in those sampled earlier (24-34 weeks, cohort 2) who later delivered with preeclampsia or SGA. In the cohort with established preeclampsia, placental and circulating SPINT2 was significantly elevated (p=0.025, cohort 3).

We next performed *in vitro* studies where we assessed the effect of inflammatory cytokines or hypoxia (1% vs 8% O_2) on SPINT2 expression in human cytotrophoblast stem cells. We also measured *Spint2* mRNA expression in placentas from a rat model of late-gestation (day 18) restricted uteroplacental perfusion (*in vivo* placental insufficiency/hypoxia model). While inflammatory cytokines did not affect SPINT2 expression, hypoxia significantly increased SPINT2 in cytotrophoblast stem cells and its expression was also significantly elevated (p=0.04) in the placental labyrinth of growth restricted rats.

In conclusion, circulating SPINT2 is increased among those with preeclampsia or SGA, though not earlier than 34 weeks' gestation. Placental SPINT2 expression was also increased in both *in vitro* and *in vivo* models of placental hypoxia.

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Reduced progesterone signalling in the peri-conception phase programs impaired immune tolerance in adult female offspring

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During pregnancy, the maternal immune environment has the capacity to influence fetal development and program altered phenotype of offspring. The pre-implantation period has emerged as the most vulnerable window in pregnancy for susceptibility to fetal programming imposed through maternal physiological and epigenetic perturbations. This results in altered embryonic gene expression, a transformed developmental program and ultimately consequences for the health of offspring. The underlying mechanisms remain to be fully elucidated. Progesterone (P4) signalling in the luteal phase and early pregnancy is a key driver of the maternal immune adaptation to pregnancy. We previously showed that luteal phase P4 signalling defect in C57Bl/6 female mice, achieved through administration of low-dose P4 antagonist RU486 on gestational days (GD) 1.5 and 3.5, compromises maternal immune adaptation causing significantly fewer uterine Foxp3+ T regulatory (Treg) cells and adverse fetal outcomes. In this study we aimed to investigate the impact of reduced peri-implantation P4 signalling-mediated immune disruption on the immune profiles of adult 16-week-old offspring. Flow cytometry was performed to assess T cell profiles in spleen, thymus and mesenteric lymph nodes from offspring of RU486-treated or carrier-treated dams. Strikingly, offspring exhibited altered T cell profiles with sex-specific differences, such that female offspring demonstrated prominent alterations in immune parameters. Most notable was a significant reduction in the proportion (P=0.0361) and total number (P=0.0343) of $CD4^+CD25^+Foxp3^+$ Treg cells in the thymus of female offspring from RU486-treated mothers. Numbers of T conventional cells ($CD4^+Foxp3^-$) were unchanged however, indicating a specific loss of Treg cell generation. These novel results demonstrate that impaired P4 signalling during the peri-conception phase is a critical determinant of immune system programming evident in adult female offspring. A less tolerogenic immune phenotype increases susceptibility to a range of inflammatory conditions and so represents a new mechanism by which peri-conception fetal programming is mediated.

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Assessment of the tocolytic nifedipine in pre-clinical primary models of preterm birth

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Objective

Spontaneous preterm birth is the leading cause of perinatal morbidity and mortality, with ~15 million babies born preterm/year globally. Nifedipine, currently used clinically to delay preterm birth, has limited efficacy. Furthermore preclinical data on nifedipine's mechanisms of actions are lacking. We hypothesise that nifedipine does not reduce the inflammation central to the pathophysiology of preterm myometrial contractions. We aimed to assess the anti-inflammatory and anti-contractile effects of nifedipine on myometrium using a novel pipeline encompassing *in vitro*, *in vivo* and *ex vivo* human and mouse models of preterm birth.

Methods

Inflammatory cytokine gene expression was evaluated (qPCR) following treatment of the myometrial cell line PHM1-41 with tumour necrosis factor-α (TNFα;0.1ng/ml) and lipopolysaccharide (LPS;100ng/ml) ±nifedipine (10μM). Myometrial contraction assays (PHM1-41 cells embedded in collagen gel) assessed the anti-contractile efficacy of nifedipine on TNFα/LPS-induced contractions. Primary human myometrial tissue (non-labouring; collected at caesarean-section) was assessed *ex vivo* (DMT

Myograph) to evaluate the effect of nifedipine on spontaneous myometrial contractions. A mouse model of LPS-induced preterm birth was developed to determine whether nifedipine delayed delivery and altered uteri expression of contraction-associated genes following birth.

Results

TNFα/LPS treatment significantly increased gene expression of pro-inflammatory cytokines interleukin (IL)-1B, IL-6 and CXCL8 in myometrial cells compared to control (n=5), and addition of nifedipine had no effect. Myometrial contraction assays demonstrated nifedipine treatment reduced TNFα/LPS-induced contractions to baseline levels (n=3). Nifedipine treatment potently diminished spontaneous myometrial contractions in human primary myometrial tissue assays compared to vehicle (ethanol) treatment (n=3). Preliminary *in vivo* findings revealed nifedipine delays LPS-induced preterm birth in 25% of mice.

Conclusion

Nifedipine reduced myometrial contractions in both human primary tissue and a cell line. Nifedipine partially prevented preterm birth in a mouse model. Given nifedipine did not reduce pro-inflammatory gene expression, we suggest nifedipine does not modulate inflammatory cytokines in the myometrium.

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Epsilon tubulin is required for fertility in male mice and has essential roles in meiosis and spermatid remodelling

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As essential components of the cytoskeleton, alpha, beta and gamma tubulin have deservedly garnered significant research attention. In contrast, their close relative, epsilon tubulin (TUBE1), has received little attention, but promises to be equally as important. Recently, we have shown epsilon tubulin is enriched during male meiosis and spermiogenesis and have identified it as a candidate target for katanin-mediated regulation of the manchette[1]. Therefore, we set out to investigate epsilon tubulin's function in spermatogenesis and understand its mechanism of action.

Using *Stra8-Cre*, we generated a pre-meiotic, male germ cell knockout of epsilon tubulin (*Tube1*$^{GCKO/GCKO}$) and found male mice to be sterile. Within these males, testicular sperm production and epididymal sperm content was reduced by 80% and 92% respectively. A closer examination of male germ cells revealed epsilon tubulin is required for spindle microtubule organization and cytokinesis during meiosis. Epsilon tubulin is also required for nuclear remodelling in haploid male germ cells, as evidenced by dysregulated manchettes in *Tube1*$^{GCKO/GCKO}$ spermatids and the formation of spermatozoa with hyper-constricted nuclei. Moreover, of the reduced numbers of *Tube1*$^{GCKO/GCKO}$ sperm produced, their swimming ability was significantly compromised, suggesting epsilon tubulin is needed for sperm tail development and/or function. Consistent with our hypothesis that TUBE1 is a target of katanin action, the phenotypes in *Tube1*$^{GCKO/GCKO}$ mice are reminiscent to those seen in our KATNAL2 and KATNB1 mutant mouse models[1, 2]. Overall, our results demonstrate epsilon tubulin is indispensable for spermatogenesis, and plays a role in multiple aspects of germ cell development.

[1]Dunleavy, J.E.M., et al., *Katanin-like 2 (KATNAL2) functions in multiple aspects of haploid male germ cell development in the mouse.* PLOS Genetics, 2017. **13**(11).

[2]O'Donnell, L., et al., *An essential role for katanin p80 and microtubule severing in male gamete production.*PLOS Genetics, 2012. **8**(5): p. e1002698.

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Comparative analysis of a newly discovered human endometrial receptivity marker in rhesus macaques and mice

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Background: Podocalyxin (PODXL) is a newly discovered endometrial receptivity marker in women; it is specifically downregulated in the endometrial luminal epithelium at receptivity to permit embryo implantation.

Aim: To determine whether endometrial PODXL expression is conserved between humans, rhesus macaques and mice for embryo implantation.

Methods: The PODXL gene, mRNA and protein sequences across species were compared bioinformatically. Immunohistochemistry and *in situ* hybridization (mouse tissues) were employed to assess uterine PODXL expression in macaques across the menstrual cycle (n = 3 per phase), and in mice across the estrous cycle (n = 4 per phase) and on pregnant d4.5, when implantation initiates (n = 4). Functional studies then investigated whether endometrial PODXL affects mouse embryo attachment.

Results: Greater sequence similarities were evident between humans and macaques than with mice. In all species, PODXL was expressed in uterine epithelial and endothelial cells. In macaques, PODXL was downregulated in the luminal epithelium in the mid-secretory phase (****P < 0.0001) when receptivity is developed, consistent with the pattern found in women. At this time, PODXL was also downregulated in shallow (****P < 0.0001) but not in deep (**P < 0.01) glands. Endothelial PODXL was

unchanged across the cycle. In mice, uterine PODXL did not vary considerably across the estrous cycle. However, at the time of implantation, PODXL was greatly reduced in the luminal epithelium especially near the site of embryo attachment. Mouse embryos failed to attach or thrive when co-cultured on a monolayer of Ishikawa cells overexpressing PODXL.

Conclusions: Endometrial PODXL is downregulated in the luminal epithelium for embryo implantation in all species examined; PODXL is confirmed to inhibit mouse embryo implantation. The rhesus macaque shares greater conservation with humans than mice in PODXL regulation, and thus represents a better animal model for studying endometrial receptivity for human fertility treatment.

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Shifting the paradigm of sperm maturation; global proteomic profiling of epididymal sperm

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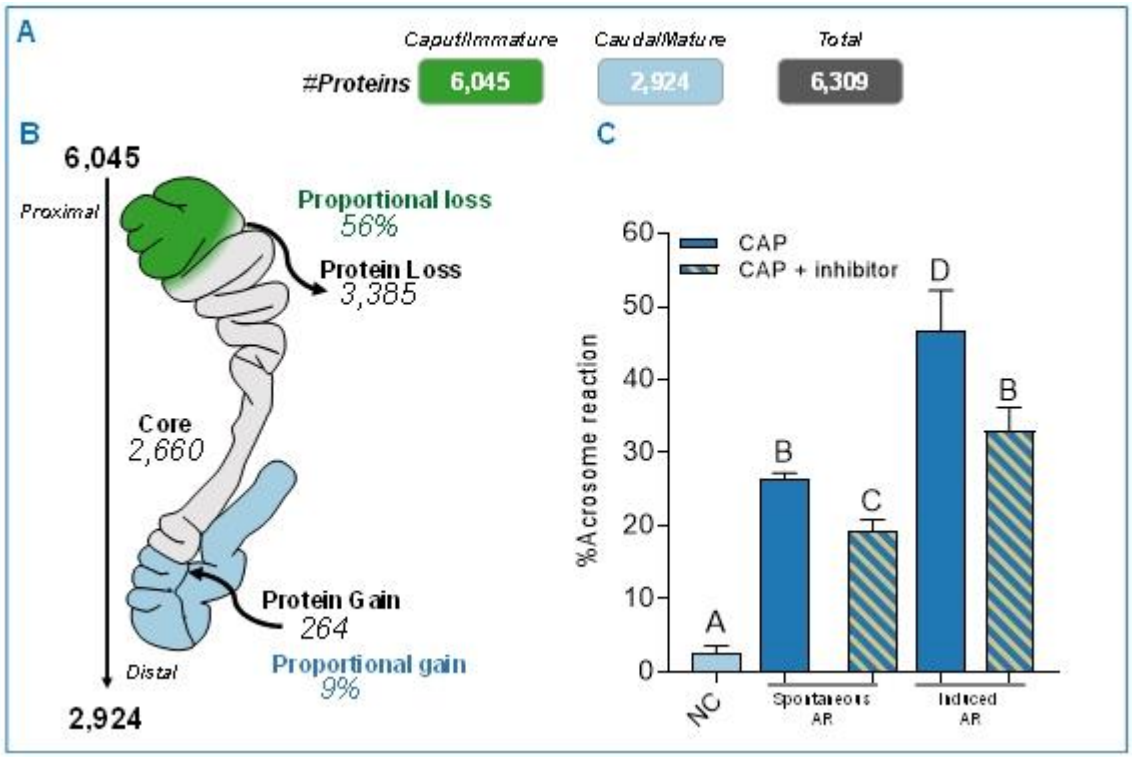
Prior to engaging in fertilisation, mammalian spermatozoa must first complete an arduous journey of functional maturation as they transit the epididymis. A distinctive feature of this process is that it occurs in the absence of *de novo* gene transcription and protein translation and is thus reliant on a substantive remodelling of the intrinsic sperm proteomic architecture, the scale of which is yet to be fully understood. Here, we have sought to define the extent of proteomic changes associated with the maturation of mouse spermatozoa using label-free quantitative mass spectrometry; reporting an unprecedented depth of coverage encompassing >6,000 proteins (Fig. A). Contrary to the long-held belief that epididymal maturation is primarily driven by the uptake of additional proteins, our data demonstrates that sperm shed over 56% of their proteins during this process, producing a refined fertilisation-competent cell (Fig. B; 9% gain). Complementing these extensive changes, the abundance of an additional 889 proteins was significantly altered as sperm transited the epididymis (≥2-fold change, $p \leq 0.05$). Furthermore, counterintuitive to the notion of gaining proteins to gain function, the reduced complexity of the sperm proteome aligned with the putative activation of key cellular functions including sperm 'motility' and 'capacitation'. In accounting for how these proteomic changes influence sperm function, we demonstrate that RHOA, a small GTPase, is acquired by maturing spermatozoa (2-fold increase), with a concomitant downregulation or complete loss of RHOA repressing proteins, including ARHGAP18, ARHGDIB, GDI-1, in mature sperm. To investigate the function of RHOA we pharmacologically inhibited its activity, resulting in a compromise to the ability of mature spermatozoa to undergo an acrosome reaction (Fig. C; ~40% reduction). These data contribute a new understanding of the mechanisms that underpin the transformation of sperm into functionally competent cells and provide a platform to identify proteins that equip sperm for fertilisation.



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Investigating the Effects of Gender-Affirming Hormone Therapy on Bone Using Pre-Clinical Models

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Gender-affirming hormone therapy (GAHT) with masculinising testosterone or feminising estradiol treatment is used to align a person's physical characteristics with their gender identity and to relieve dysphoria and depression. Sex steroids are critical for bone growth during puberty and bone maintenance in adulthood. Surprisingly however, the impact of GAHT on bone health in transgender people is poorly studied. As such, there is an unmet clinical need to definitively clarify the effects of sex steroids on bone in transgender people to prevent long term risks of bone disease. A critical step to understanding this is to examine how the circulating levels of testosterone and estradiol interact with the local synthesis of these steroids within bone to regulate bone cell function and strength. We will present data from preclinical mouse models which mirror GAHT in adolescent and adult male-to-female transitioning humans. Murine models are advantageous as they provide essential information relating to the testosterone and estradiol concentrations within bone, as well as bone microstructure, density, breaking strength, and cellular activity that are unable to be obtained from humans.

The most striking observation in this ongoing study is in the model of pubertal male-to-female transition whereby puberty is first arrested by orchidectomy followed by estradiol treatment. Estradiol treatment is sufficient to restore the loss of cortical and trabecular bone associated with puberty suppression, but does not restore bone size, consistent with the periosteal apposition of bone during puberty in males being mediated via testosterone action. The impact of these structural changes on bone strength and cell metabolism as well as the local concentrations of sex steroids within bone following GAHT are currently being assessed.

The findings from this study will provide significant insight into the actions of sex steroids within bone which will assist in informing treatment for maintaining bone health in transgender people undergoing GAHT.

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Gender affirming hormonal therapies for trans and gender diverse adolescents

Michele O'Connell[1]

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Nationally and internationally, increasing numbers of trans and gender diverse children and adolescents are presenting for care. In response, gender affirming hormonal therapies that seek to align physical characteristics with an individual's gender identity are increasingly being employed in adolescents with gender dysphoria. Depending on a young person's identity and goals, these therapies may aim to achieve full pubertal suppression, modulation of the effects of endogenous pubertal sex hormone and/or development of secondary sex characteristics congruent with their affirmed gender. Endocrine practice in this area is still relatively novel and while short-term outcomes are encouraging, longer-term data from prospective longitudinal cohorts of adolescent are still lacking. This presentation will provide an overview of current pharmacological options and potential treatment pathways, reported outcomes and clinical challenges in the management of trans and gender diverse adolescents.

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Psychological Challenges for Transgender Patients

Emerson Osterberg[1]

1. Healthy Minds, Happy Kids, Parramatta, NSW, Australia

One in five transgender people face discrimination from medical professionals each week which leads to them not seeking further medical intervention. The current presentation aims to provide information about the mental health trends of the transgender and gender diverse population, exploring factors that lead to better mental health outcomes for this marginalised group. A conversation about what changes we need to make in both our professional practice and our day-to-day lives to create a more welcoming and inclusive environment for transgender and gender diverse people is explored.

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Clinical Aspects of Bone Health in the Transgender Population

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There are increasing numbers of transgender (otherwise known as trans) which includes people who are undergoing gender-affirming hormone therapy. As many individuals undergo testosterone (in trans men) or estradiol (in trans women) therapy lifelong, long-term effects are important. Given the importance of sex steroids in attaining and maintaining peak bone mass and bone health and strength in adulthood, hormonal fluctuations may upset this balance.

Previous studies in bone health in trans people have used predominantly 2-dimensional areal bone mineral density (aBMD) and suggest that trans people at baseline appear to have lower aBMD than the general population. Lifestyle and environmental factors likely contribute with lower vitamin D levels, higher rates of smoking and lower muscle strength observed, particularly in trans women. Lower estradiol concentrations are associated with a greater decrease in aBMD over time. Only 1 study suggests fracture risk is higher in older trans women compared with age-matched reference men, but no increase was seen in trans men. It is unclear whether this increased fracture risk is related to baseline lower aBMD.

A cross sectional study examining 3-dimensional volumetric bone mineral density (vBMD) has found lower vBMD in trans women compared to cisgender male controls and higher cortical porosity. Trans men had higher vBMD relative to cisgender female controls.

Overall, trans women may not be protected from microstructural deterioration by estradiol therapy, and attention should be made to ensure adequate estradiol concentrations are achieved. Emphasis should also be made on lifestyle factors including encouraging exercise, optimisation of vitamin D and smoking cessation. No adverse effects on bone microstructure appear to occur in trans men. Further long-term longitudinal controlled studies are required, particularly in non-binary individuals using low doses of gender-affirming hormone therapy or in individuals who have previously received GnRH analogues.

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Being transgender: a community member's perspective

Clare Headland[1]

1. Melbourne, VIC, Australia

Available Soon

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Regulatory T cells facilitate maternal vascular adaptations to pregnancy

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Preeclampsia is an important cause of maternal and perinatal morbidity and mortality, and increases the susceptibility of the mother and offspring to cardiovascular disease later in life. In preeclampsia, a deficiency in regulatory T (Treg) cells has been observed. Treg cells prevent maternal immune rejection of the fetus, and suppress inflammatory activation. We have shown they also contribute to uterine vascular function in pregnant mice (Care et al Hypertension 2018), consistent with emerging roles in systemic vascular homeostasis. However, the role of Treg cells in spiral artery remodelling in early placental development remains unclear. Uterine natural killer (uNK) cells are a specialised subset of innate immune cells, with a pivotal role in spiral artery remodelling during pregnancy. Although there is evidence of Tregs and NK cells communicating in the peripheral organs, whether and how they interact within the uterus during pregnancy is unknown. We hypothesise that Treg cell deficiency in early pregnancy will alter uterine natural killer cell (uNK) abundance, leading to impaired spiral artery remodelling and fetal growth restriction. Using transgenic *Foxp3-DTR* mice we can elicit selective depletion of FOXP3+ (Treg) cells to investigate the role of Treg cells in maternal vascular adaptations to pregnancy. In mid-pregnancy Treg cell depletion led to perturbed uterine artery function. Decidual spiral artery remodelling was impaired, evidenced by a smaller artery lumen area and smooth muscle actin retention, leading to a reduction in fetal weight in late gestation. Moreover, the abundance of uNK cells in the decidua was decreased in Treg-depleted mice compared to controls. However, administration of exogenous wild-type Treg cells restored uterine artery function, spiral artery remodelling and uNK cell abundance, demonstrating a causal role for Treg cell deficiency. We demonstrate an essential role for Treg cells in fetal growth and uteroplacental vascular function. Given the severe implications of preeclampsia on the future health of the mother and her offspring, investigation of therapeutic strategies targeting Treg cells may offer a promising target for intervention.

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Oocyte-cumulus cell bidirectional communication impacts oocyte quality

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Mammalian oocytes grow and mature in a mutually dependent relationship with adjacent somatic cells. The oocyte actively regulates cumulus cell differentiation and function by secreting soluble oocyte-secreted factors which act on cumulus cells in a paracrine manner. Here we investigated the molecular mechanisms by which two oocyte-secreted factors, cumulin and BMP15, regulate oocyte maturation and cumulus-oocyte cooperativity. Immature antral-follicle mouse cumulus-oocyte complexes (COCs) were matured in vitro ± 20ng/mL cumulin or BMP15 (n= 3-8 biological replicate experiments). Thereafter oocytes and cumulus cells were separated and underwent morphological, metabolic, and proteomic analyses. Global analyses (proteomics and hyperspectral analysis of autofluorophores) revealed proteomic and metabolic profiles which discriminate cumulin- and BMP15-treated oocytes/cumulus cells from untreated cells and from each other. In oocytes, proteomic data showed significant upregulation of proteins involved in nuclear function in response to cumulin. In cumulus cells, proteomic data showed marked

upregulation of a variety of metabolic processes (mostly anabolic) in response to cumulin and BMP15, including lipid, nucleotide and carbohydrate metabolic processes, while mitochondrial metabolic processes were downregulated. These mitochondrial changes were supported by transmission electron microscopy showing marked morphological changes to cumulus mitochondria and endoplasmic reticulum, while mitotracker staining showed a 17% decrease in mitochondrial content ($p<0.05$) in cumulin-treated cumulus cells. In support of reduced mitochondrial number, the hyperspectral unmixed data showed significantly lower NAD(P)H levels and lower REDOX state ($p<0.05$). Furthermore, cellular and mitochondrial respiration (oxygen consumption rate) was significantly lower ($p<0.05$) in cumulin-treated COCs. Despite this, mass spectrometry quantification of nucleotides demonstrated that overall energy metabolites were in balance since most cellular energy pathway metabolites, particularly ATP, remained similar in untreated and treated COCs. Collectively, these data demonstrate that oocyte-secreted factors remodel COC metabolism during oocyte maturation in preparation for ensuing fertilization and embryonic development, and this is associated with oocyte developmental competence.

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Oral Metformin: the first disease modifying drug for preeclampsia

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Preeclampsia is a morbidity and mortality risk for both mother and the unborn baby. There is only one drug – aspirin - which prevents preeclampsia (relative risk reduction of only 18%). And once diagnosed, there are no drugs that can slow disease progression.

Metformin is currently used for glycaemic control in gestational diabetes. In bench to bedside studies, our team has discovered metformin may have the ability to quench disease severity in those diagnosed with preeclampsia.

The Translational Obstetrics Group set up a laboratory drug screening pipeline which utilises multiple assays and tests drugs for their ability to 1) decrease placental release of sFlt-1 and other anti-angiogenic factors, and 2) decrease endothelial dysfunction in maternal blood vessels (1 and 2 are thought to be central to the pathogenesis of preeclampsia).

Using our laboratory drug screening pipeline, we reported metformin may be a promising drug to treat or prevent preeclampsia (Brownfoot et al AMJOG 2016).

We translated this idea to the clinic and have just completed a large trial in South Africa (PI2 Trial). We randomised 180 participants with preterm preeclampsia (26-32 weeks gestation) to placebo or 3 grams of oral metformin. Those in the metformin arm had a prolong pregnancy of 7.6 days (primary outcome). Importantly, there was a reduced length of neonatal admission post birth (which may reflect better health at birth). We are now implementing The PI3 Trial, which will randomise 500 participants. If our findings are validated, metformin may be the first disease modifying drug for preeclampsia. The potential for clinical impact is significant.

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Omega-3 Supplementation to Prevent Preterm Birth

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Preterm birth (PTB), defined as delivery before 37 completed weeks' gestation, represents one of the greatest burdens to perinatal health. For babies born preterm, increased rates of respiratory, cardiovascular, metabolic, and neurodevelopmental complications have been observed in the short-, medium-, and long-term.

Over the last three decades, there is growing consensus that omega-3 (n-3) supplementation may reduce the rate of PTB. The greatest benefits of n-3 supplementation on PTB risk reduction have been observed in pregnant women with low n-3 status or intake. In contrast, n-3 supplementation in pregnant women replete with omega-3 may increase their risk of PTB.

Literature has suggested that the effect of n-3 supplementation may vary between mothers based on their baseline omega-3 status. As such, a broad-based approach to n-3 supplementation may not be appropriate. Instead, evaluation of maternal omega-3 status prior to initiation of n-3 supplementation is likely to be of value.

Limitations in current methods of evaluating maternal n-3 status demonstrates the need for the development of non-invasive and affordable prediction tools to identify mothers with low or sufficient n-3 status to ultimately guide clinical decision making around n-3 supplementation in pregnancy. In addition, future research should determine the optimum regime for n-3 supplementation to maximise the reduction of preterm birth. If n-3 supplementation could be optimised for Australian women, there is the potential to save up to $100M per year by preventing up to 856 early preterm births.

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Targeting N-cadherin during ovulation- a non-hormonal contraceptive

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Worldwide, over 225 million women have an unmet need for modern contraception. Unmet need accounts for 74 million unplanned pregnancies and 70,000 maternal deaths yearly. Adverse side-effects of hormonal contraceptives is the most frequent reason for discontinued or non-use. There is an acute need for new contraceptives that do not alter normal endocrine profile and offer wider contraceptive choices.

Selectively blocking ovulation without affecting ovarian hormones is an ideal approach to contraceptive development that minimises/ eliminates side effects.

We developed a high-throughput approach for screening drug libraries for potential ovulation blocking capacity using automated assessment of cumulus oocyte complex (COC) adhesion to extracellular matrix *in vitro*. Two small molecules CRS-006 and LCRF-0006, N-cadherin antagonists, were identified as "hits" that potently and dose dependently inhibited COC adhesion to fibronectin. During COC *in vitro* maturation, both N-cadherin antagonists severely inhibited COC expansion and oocyte meiotic resumption with concomitant loss of b-catenin and E-cadherin at the oocyte cell membrane. Profiling of the transcriptional response to N-cadherin inhibition identified targets of b-catenin and YAP1 pathways were dysregulated indicating that in COCs these pathways are dependent on N-cadherin action. *In vivo*, treatment with N-Cadherin antagonist CRS-066 significantly reduced ovulations (11 vs 26 oocytes/ovary; p-5.8*10^-6) compared to controls in mice. Ovarian histology and immunofluorescence revealed structural dysgenesis of follicles, with disorganised granulosa and cumulus cell layers. In particular, connections between cumulus corona radiata cells and the oocyte was disrupted. This was not due to LH-pathway downregulation as *Lhcgr* and downstream signaling remained intact. Again, the transcriptome analysis indicated that mechanistically, N-cadherin antagonism caused dysregulation of Hippo/YAP and b-catenin mechanosensitive pathways in vivo. Overall, this study establishes a robust proof-of-concept to develop a unique high-throughput model for screening drugs for contraceptive potential; and demonstrates a critical role of N-cadherin in oocyte-cumulus signaling during follicle development and ovulation.

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Ovarian cell metabolism at ovulation is correlated with oocyte competence and impacted by obesity and aging

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Aging and obesity are two major causes of female subfertility. Mitochondrial dysfunction has been proposed as an underlying cause however, there is little holistic understanding of metabolism dynamics with the ovarian follicle. To address this, we used real time metabolic analysis (Seahorse XFe96) to map energy metabolism dynamics (mitochondrial respiration, glycolysis and fatty acid oxidation) in mouse granulosa cells and cumulus-oocyte complexes (COCs) across a detailed timecourse in the lead up to ovulation. ATP production in granulosa cells was increased at 8hrs after hCG and maximal at 12hrs post-hCG, due to increased mitochondrial respiration, glycolysis and fatty acid oxidation. In stark contrast, total ATP production of COCs showed a rapid increase at 4 hours of hCG, and then gradually decreased until ovulation. The effects of obesity and aging on ATP production was examined in preovulatory follicles. Mitochondrial respiration, but not glycolysis, was reduced in granulosa cells of obese mice; and females that were both obese and reproductively old mice showed a marked decrease in both mitochondrial respiration and glycolysis. Similar metabolic alterations were observed in the ovarian stromal faction. To translate these findings, the metabolic profile of granulosa cells was measured in a cohort of 130 women undergoing IVF/ICSI cycles, and correlated with clinical parameters and cycle outcomes. Increased age, BMI and total FSH dose resulted in significant alterations in granulosa cell metabolic profile. Further, the follicular metabolic profile was significantly correlated with IVF outcomes. Overall, we demonstrate dynamic increases in multiple energy metabolism pathways in response to the LH-surge in key ovarian follicle cells. Mitochondrial respiration and glycolysis were impaired with obesity and aging, in mice and women, providing new insights into the cellular mechanisms of subfertility, by demonstrating specific metabolic perturbations that are associated with poor oocyte quality.

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Modulation of the oocyte epigenome by a mitochondrial fission protein

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Mitochondrial biogenesis occurs throughout oocyte growth, when the number of mitochondria increases from ~1000 to ~500,000 through tightly controlled processes of mtDNA replication and ultimately fission of mitochondria via the GTPase DRP1 (dynamin-related protein 1). In addition to providing increased capacity for ATP production, mitochondrial fission in other cells regulates mitochondrial quality and key cellular functions, such as redox balance. However, in oocytes it is not known whether mitochondria perform additional functions and how these impinge upon embryo reprogramming and developmental potential.

To investigate this, we developed an oocyte-specific *Drp1* Cre-*loxP* knockout (KO) system in mice to irreversibly remove DRP1 protein at the primordial follicle stage and comprehensively characterised oocyte quality including mitochondrial function, transcriptomics, proteomics and epigenetic profile. We find that *Drp1* KO oocytes are able to grow and meiotically mature, have normal ATP concentrations but reduced mitochondrial membrane potential and moderately increased mitochondrial reactive oxygen species levels compared to controls. Analysis of *Drp1* KO oocytes reveals dramatic changes to the transcriptome and proteome, as well as disrupted levels of epigenetic marks in oocyte nuclei. To determine the impact of these changes on developmental competence, *Drp1* KO oocytes were fertilised with WT sperm such that embryos expressed DRP1 following zygotic genome activation but had inherited mitochondria from the DRP1-deficient oocyte. Remarkably, *Drp1* KO oocytes fertilized normally but embryos showed high rates of developmental failure after day 13 of gestation. To determine whether developmental failure is due to disrupted mito-nuclear communication, pronuclear transplantation experiments were conducted. These showed that placing the nuclear material from *Drp1* KO oocytes into wild type cytoplasm did not rescue the developmental

failure. These studies are the first reveal a regulatory role of mitochondria, specifically via DRP1, in the establishment of the maternal epigenome during oocyte growth, which is critical for normal fetal development.

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Cyclin A2 supports faithful chromosome segregation in the oocyte to embryo transition

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Publish consent withheld

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PRC2 establishes H3K27me3 at developmental genes in growing oocytes and regulates offspring development rate

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Epigenetic modifications modulate cell differentiation partly by regulating transcription of developmental genes. While it has been proposed that epigenetic programming of germ cells is critical for offspring development and health, the mechanisms are poorly understood. As extensive evidence suggests that environmental factors, including drugs or diet, can alter germline epigenetic programming, understanding these mechanisms is essential. Polycomb Repressive Complex 2 (PRC2) catalyses the epigenetic modification, H3K27me3, to repress developmental genes in many tissues. Using genetic mouse models to delete PRC2 function in the oocyte, we examined how H3K27me3 establishment is regulated in growing oocytes. We identified a key window of transient PRC2 activity that regulates establishment of H3K37me3 at developmentally important genes in growing oocytes. Oocyte-specific deletion of the essential PRC2 subunit, *Eed*, de-repressed 343 genes (DEGs), primarily involved in neurogenesis and development, in fully grown Germinal Vesicle (GV) oocytes. Importantly, many of these genes contained H3K27me3 in human GV oocytes suggesting this PRC2 activity is conserved in humans. Comparison of the DEGs with classically and non-canonically imprinted genes strongly indicated that EED regulation of these DEGs in growing oocytes represents a novel function for PRC2 in regulating maternal inheritance. Consistent with this, post-implantation offspring from *Eed*-null oocytes were initially developmentally delayed but exhibited increased placental weights and catch-up growth ultimately resulting in post-natal overgrowth compared to genetically identical controls. Significantly, *de novo* germline mutations in human EED/EZH2 result in Cohen-Gibson/Weaver Syndromes, characterised by overgrowth, skeletal abnormalities and learning deficits. Our work identifies a novel link between EED-dependent oocyte epigenetic programming and offspring development and strongly indicates that this activity is conserved in human oocytes. Understanding these processes is critical for determining epigenetic inheritance, and how exposure to clinically relevant EZH2 or EED inhibiting drugs may impact on oocyte epigenetic programming, and subsequent health and development of the next generation.

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Checkpoint inhibitor immunotherapy diminishes oocyte number and quality: consequences for fertility of female cancer survivors

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Conventional cytotoxic cancer therapies exert permanent damage to the ovary, hence, loss of fertility is a major concern for female reproductive-age cancer survivors. However, the landscape of cancer therapies is rapidly changing, with attention shifting to more personalised, targeted treatments. Immunotherapies, like checkpoint inhibitors anti-PD-1, anti-PD-L1 and anti-CTLA-4, harness the immune system to kill tumour cells. They are increasingly becoming a standard of care for many tumour types, including in the curative setting. But, their impacts on ovarian function and fertility are unknown.

We evaluated the effect of anti-PD-1, anti-PD-L1 and anti-CTLA-4 antibodies on the ovary using tumour-bearing and tumour-free mouse models. In tumour bearing mice, combination immune checkpoint inhibition (anti-PD-1 + anti-CTLA-4) increased intra-ovarian CD4+ (1003±76 versus control 632±53; p=0.007) and CD8+ T cells (1072±129 versus control 628±58; p=0.02) and TNF-α cytokine production. Disruptions to folliculogenesis and ovulation were observed, with a significant 117% increase in antral follicle atresia (90±20.7, versus control 41.4±10.5; p=0.04) and reduction in corpora lutea (2.4±0.4, versus control 5±0.4, p=0.003), indicating reduced ovulations.

Profound and permanent impacts to ovarian function were also detected in tumour-free mice. PD-L1 or CTLA-4 blockade induced significant depletion of the ovarian reserve of primordial follicles by 43% and 38% respectively (1031±161 and 1116±247 versus control 1809±167, p<0.05) after 21 days. Notably, in women, primordial follicle depletion is associated with early loss of fertility and premature menopause. Furthermore, the number ovulated oocytes was significantly reduced following anti-CTLA-4 treatment (25±3, versus control 34±2; p=0.03), whereas anti-PD-L1 increased the number of fragmented/dead oocytes (8±2, versus control 2±1; p=0.03).

Collectively, these data demonstrate that immune checkpoint inhibitors have the potential to impair both the immediate and future fertility of young women. Hence, fertility preservation should be strongly considered for women receiving these immunotherapies, and investigation of preventative strategies must be prioritised in future studies.

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Molecular modelling using a PCOS mouse model in combination with androgen receptor knockout mouse models (ARKO) to unravel PCOS pathogenesis

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Polycystic ovary syndrome (PCOS) is a common heterogeneous disorder characterized by endocrine, reproductive and metabolic dysfunction. The etiology of PCOS is poorly understood, however, hyperandrogenism is a key diagnostic feature and evidence supports a role for androgen receptor (AR) mediated actions in PCOS pathogenesis. Aberrant AR signaling in adipose tissue and muscle are proposed as being implicated in the manifestation of PCOS, but their significance and the precise AR signaling mechanisms involved remain unclear. This study investigated the role of AR signaling in white adipose tissue (WAT), brown adipose tissue (BAT) and skeletal-muscle in the development of PCOS traits. We exposed wildtype (WT), global androgen receptor knockout (ARKO) and skeletal muscle specific ARKO (SkMARKO) mice to dihydrotestosterone (DHT) to induce PCOS traits. ARKO ($AR^{-/-}$) WAT/BAT were transplanted into WT ($AR^{+/+}$) DHT-induced PCOS females, and WT WAT/BAT were transplanted into ARKO DHT-induced PCOS females. After 12 weeks of DHT exposure, reproductive and metabolic PCOS traits were assessed. DHT induced key reproductive and metabolic PCOS traits in WT females. SkMARKO mice treated with DHT displayed a similar phenotype to DHT-treated WT females, with full development of reproductive and metabolic PCOS features. Transplantation of ARKO WAT/BAT into DHT-treated WT females prevented the development of some metabolic PCOS features, as mice displayed significantly lower body weights (P<0.05), decreased visceral adiposity (P<0.05), and smaller adipocyte size (P<0.05) compared to DHT-treated WT females with sham surgery. However, reproductive PCOS traits were not ameliorated by ARKO WAT or BAT transplantation. Furthermore, DHT exposed ARKO female mice transplanted with WT WAT/BAT did not develop PCOS traits. In summary, WAT and BAT, but not skeletal muscle, are key sites of AR-mediated actions involved in the development of PCOS-associated metabolic traits. These findings support targeting adipocyte AR-driven pathways in future research for the development of novel therapeutic interventions for PCOS.

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Progesterone receptor regulation of ovulation via respiration, glycolysis, fatty-acid oxidation and cell-specific signaling

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Anovulation is the leading cause of female infertility, therefore it is essential to identify the mechanisms regulating ovulation to gain insight into the underlying causes of female infertility and develop novel therapeutic interventions. Progesterone receptor (PGR), a steroid-dependent transcription factor expressed in ovarian granulosa cells, is obligatory for ovulation in mammals. We investigated effector pathways activated by PGR in order to identify those that are crucial for oocyte release. We hypothesised that PGR may upregulate ATP production to fuel ovulatory processes. To address this we investigated major metabolic pathways (mitochondrial function, glycolysis and fatty-acid oxidation) in granulosa cells of mice null for PGR (PRKO). Using real-time cell metabolism assays we found that surprisingly ATP production via mitochondrial respiration or glycolysis was not PGR-regulated. Supporting these observations, treatment of mice with rotenone, an inhibitor of Complex I reduced granulosa cell mitochondrial respiration but this did not impair ovulation. In parallel, we performed RNA-seq analysis of both isolated granulosa cells and the GC-depleted ovarian stromal tissue to identify differentially expressed genes in PRKO vs WT mice. Ingenuity Pathways Analysis elucidated a number of interesting pathways that were dysregulated in the two ovarian cell compartments. Of note, 'lipid metabolism' was dysregulated in granulosa cells of PGR-null mice, and a network of adipose-related transcription factors, *Bcl11b, Pparg, Hnf4a* and *Zbtb16,* was identified as PGR-regulated with gene expression induced during ovulation. Additionally, *Cpt1a* and *Cpt1b*, the rate-limiting enzymes for fatty-acid oxidation were PGR-regulated in granulosa cells. These findings advance our understanding of steroid-receptor regulation of metabolism, demonstrating that PGR does not regulate mitochondrial respiration or glycolysis in granulosa cells but activates alternative pathways for lipid regulation during ovulation. Additionally, RNA-seq analysis of both granulosa and stromal cells has revealed intriguing PGR-regulated gene networks that shed light on the intercellular mechanisms regulating ovulation.

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Diverse Roles of the Androgen Receptor in Breast and Prostate Cancer

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Since the introduction of androgen deprivation therapy (ADT) by Huggins and colleagues some 80 years ago, it has become the mainstay strategy for treatment of prostate cancer. ADT specifically targets the androgen receptor (AR). Since the cloning of the human AR in the late 1980's extensive evidence has accumulated for its oncogenic role in prostate cancer, leading to potent new hormone therapies to target the AR. Unfortunately, ADT remains a blunt clinical weapon as AR activity influences the health and function of most body tissues. Side effects associated with ADT adversely affect quality of life in patients and can contribute to drug non-compliance. Moreover, while new hormone therapies developed over the past decade for the treatment of metastatic hormone sensitive prostate cancer prolong life, resistance to these agents is inevitable as tumours evolve to reactivate AR and regulate expression of genes that promote tumour growth and survival. Smarter therapeutic strategies are needed to achieve more durable responses and improve quality of life for patients actively on ADT. More recently, we and others have investigated reprograming of AR from an oncogenic to a more normal, pro-differentiating factor rather than abolishing its activity altogether, thus driving AR toward better outcomes in prostate cancer.

Resistance to estrogen receptor alpha (ER) target therapies is the major cause of breast cancer death. Therapeutic engagement of steroid receptors that impinge on, but do not ablate, ER signalling is an emerging new treatment strategy. The AR is expressed in the majority of breast tumours and represents an exceptional therapeutic target, especially as a range of drugs including AR agonists, antagonists and selective AR modulators (SARMs) are available. Interest in targeting AR for treatment of breast cancer has escalated over the past decade. Despite clinical correlations and preclinical studies supporting a protective role for AR in breast cancer, especially in ER-positive disease, enthusiasm was largely directed toward antagonizing AR with anti-androgenic drugs used to treat men with prostate cancer, a disease definitively driven by oncogenic AR activity. While some pre-clinical studies supported use of an AR antagonist for ER-positive breast cancer, this strategy has had minimal success in clinical trials. Our most recent preclinical studies using a diverse range of *in vivo* breast cancer models (Hickey et al, *Nature Medicine* 2021) provides compelling evidence for (i) AR being a tumour suppressor in ER+ breast cancer, and (ii) an AR agonist, not an antagonist, treatment strategy being the optimal therapeutic approach.

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Clinical factors affecting insulin dose requirements during treatment of prednisolone-induced hyperglycaemia

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Background: Prednisolone is a semi-synthetic glucocorticoid hormone that commonly causes hyperglycaemia in hospitalised patients, which is associated with increased morbidity and mortality. Weight-based insulin regimens are recommended to treat prednisolone-induced hyperglycaemia, but many patients remain hyperglycaemic due to wide variability in insulin dose requirements.[1] To inform insulin dosing, we investigated which clinical characteristics are associated with daily insulin requirements in patients prescribed prednisolone.

Methods: In this prospective study, 50 adult medical inpatients with an acute inflammatory illness and prescribed a morning dose of prednisolone ≥20 mg/day with hyperglycaemia (one finger-prick blood glucose level (BGL) >15 mmol/L or two BGL >10 mmol/L within 24 hours) received an intravenous Actrapid insulin infusion for 24 hours to determine their daily insulin requirements. Hourly flash glucose monitoring was performed with Freestyle Libre Device (Abbott). The daily insulin dose required to attain an average flash glucose concentration of 8.0 mmol/L was calculated. Associations between clinical variables and daily insulin dose were examined using linear regression.

Results: The participants age was 69 ± 10 years, prednisolone dose was 34 ± 10 mg, HbA1c was 7.7 ± 2.0%, 77% had known type 2 diabetes and 30% were female. In univariate analysis, weight was weakly but significantly associated with daily insulin dose (Table). Other variables associated with daily insulin dose were HbA1c, sex, prior diabetes treatment and peak glucose before commencing insulin; prednisolone dose was not. In multivariate analysis HbA1c, weight, sex, diabetes treatment and diabetes status were independently associated with daily insulin dose ($R^2 = 0.65$, $p<0.001$).

Conclusions: Combining HbA1c, sex, diabetes status, diabetes treatment and weight explains an additional 54% of the variability in daily insulin dose in patients with hyperglycaemia while prescribed prednisolone, compared to weight alone. Incorporation of these additional clinical characteristics into individualised insulin algorithms will refine insulin dosing for prednisolone-induced hyperglycaemia.

Table

	Univariate		Multivariate	
	R^2 value	P value	β co-efficient	P value
HbA1c	0.39	<0.001	0.47	<0.001
Weight	0.11	0.024	0.29	0.005
Sex	0.11	0.025	0.20	0.055
Peak Glucose	0.30	0.001	0.39	0.312
Diabetes	0.06	0.11	0.29	0.04
Diabetes Treatment	0.23	<0.001	0.54	0.003
CRP	0.12	0.47	-	-
Prednisolone dose	0.16	0.36	-	-
Diagnosis	0.001	0.81	-	-

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An algorithm to guide radioactive iodine treatment.

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Background: Personalized recurrence risk ideally guides treatment with radioactive iodine (RAI) in differentiated thyroid cancer. 2015 ATA guidelines provide some risk stratification to aid treatment decisions but is not comprehensive. The aim of our study was to develop an algorithm for RAI treatment based upon personalized recurrence risk calculated using ATA risk features combined with histological extra-thyroidal extension (ETE) and post-operative serum thyroglobulin (sTg).

Methods: Retrospective multivariable analysis of 1117 patients who received RAI following thyroidectomy at a quaternary centre in Australia between 2008-2018, with a sTg (TSH > 30mIU/L). Prospectively collected data included age, gender, histology and AJCC staging. ATA risk was calculated retrospectively.

Results: In intermediate and high ATA risk there was no difference in risk of recurrence between 4GBq or 6 GBq activity of RAI ($p = 0.10$). Independent of ATA risk, patients with a sTg > 10ug/L had a worse progression free survival (PFS) than those with sTg < 10ug/L ($p < 0.001$). ETE stratified by 4 histological categories was significantly associated with PFS ($p < 0.001$). On multivariate analysis gross ETE, sTg > 10 ug/L, multifocal papillary thyroid cancer (PTC), follicular variant PTC and follicular thyroid cancer were associated with synchronous metastasis. Our algorithm, with 5 recommended activities was validated on 218 patients treated with RAI in 2017-2018 correctly recommending RAI activities for patients in ATA low and high risk groups and for those with synchronous metastasis.

Conclusions Our algorithm extends ATA risk stratification by including sTg and ETE which we found to be independent predictors of recurrence and synchronous metastasis in DTC. We have developed an online RAI decision support tool to provide evidence based treatment guidelines, reducing unnecessary RAI treatments while confidently treating patients with high risk of metastatic disease.

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Corticosteroid-binding globulin deficiency independently predicts mortality and is associated with norepinephrine requirements in septic shock.

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Surveillance improves outcomes for *SDHB* mutation carriers: a multi-centre study

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Background

Succinate dehydrogenase type B (SDHB) mutation carriers are at risk of phaeochromocytoma and paraganglioma from a young age. It is widely recommended that patients enter a lifelong screening program to detect tumours, as metastatic disease is associated with high mortality.

Objective

The purpose of this study was to describe screen-detected tumours in *SDHB* mutation carriers enrolled in a screening-program, to assess predictors of disease diagnosis, and to compare outcomes in screen-detected tumours to probands.

Methods

This was a multi-centre study of clinical data collected by both retrospective and ongoing prospective follow-up in genetics clinics at Royal North Shore Hospital (RNSH), Prince of Wales Hospital (PoWH) and the Royal Hobart Hospital (RHH). Probands were defined as the first individual in a family to be diagnosed with a genetic condition after presenting with a tumour. Annual clinical assessment of catecholaminergic symptoms, blood pressure and biochemical measurements of plasma metanephrines or urinary catecholamines were included in the surveillance protocols of all three centres. RNSH and PoWH followed the Cancer Institute NSW guidelines for two yearly MRI base of skull to coccyx imaging surveillance (https://www.eviq.org.au) while RHH surveillance consisted of second yearly imaging with ultrasounds neck and abdominal and after age 18 with four yearly fluorodeoxyglucose (18F) positron emission tomography/computed tomography (18F-FDG PET/CT).

Results

182 *SDHB* mutation carriers from 59 families undergoing routine surveillance were assessed. Median age at first surveillance was 33 years (range 1 to 81 years). There were 33 probands and 149 non-probands. Median duration of follow up was 6.0 ± 0.4 years. Tumours were detected in 30 of 149 (20%) non-probands undergoing screening (age range 9-76 y). Male gender and young age were associated with detection of tumours. Patients with screen-detected tumours were less likely to be associated with metastatic disease compared to probands. Estimated 10-year metastasis-free survival was 66% for probands and 84% for non-probands with screen-detected tumours (p=0.0268). Screen-detected tumours were smaller than those in probands (median 28 ± 4.2 mm versus 45 ± 8.3 mm respectively, p=0.004). Tumour size 40mm was associated with progression to metastatic disease (OR 16.0, 95% CI 2.3-177.5, p=0.003). Patients with screen-detected tumours had lower mortality compared to probands: 10-year overall survival was 79% for probands and 100% for non-probands (p=0.0286).

Conclusion

This is the largest Australian study to describe screen-detected tumours and outcomes for *SDHB* mutation carriers in a surveillance program. Patients with screen-detected tumours had smaller tumours, reduced risk of metastatic disease and lower mortality compared to probands. Tumour size 40mm was associated with increased risk of metastatic progression. Our results suggest that *SDHB* mutation carriers should undertake surveillance to improve clinical outcomes.

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Effects of estradiol on bone density and fat mass in men undergoing androgen deprivation therapy for prostate cancer: a randomised placebo-controlled trial

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Objective: Indirect evidence suggests that the effects of testosterone on bone and fat in men are dependent on aromatisation to estradiol (E2). No controlled study has examined this hypothesis in the absence of testosterone.

Design: 6-month randomised, placebo-controlled trial. We hypothesised that E2 would increase distal tibia total volumetric bone mineral density (vBMD) and reduce fat mass.

Methods: 78 participants receiving androgen deprivation therapy (ADT) for prostate cancer were randomised to 0.9mg topical E2 per day, or placebo. High-resolution peripheral quantitative CT was performed at baseline and study end. Body composition and areal bone mineral density (aBMD) were measured by dual energy x-ray absorptiometry at baseline, month 3 and month 6. At each visit sex steroids were measured by liquid chromatography tandem mass spectrometry, and serum beta carboxyl-terminal type 1 collagen telopeptide (CTX) and pro-collagen type 1 amino-terminal propeptide (P1NP) were measured by electrochemiluminescence.

Results: Serum E2 increased in the E2 group over 6 months compared to placebo, mean adjusted difference (MAD) 207pmol/L (95%CI 123–292), p<0.001. We observed no effect on distal tibia total vBMD, MAD 2.0mgHA/cm^3 (95%CI -0.8–4.8), p=0.17. E2 increased distal radius cortical vBMD, MAD 14.8mgHg/cm^3 (95%CI 4.5-25.0), p=0.005, and aBMD of lumbar spine, MAD 0.02g/cm^2 (95%CI 0.01-0.03), p=0.01, and ultradistal radius, MAD 0.01g/cm^2 (95%CI 0.00–0.02), p=0.01. E2 reduced CTX, MAD -224ng/L (95% CI -305 to -143), p<0.0001, and P1NP, MAD -13mcg/L (95% CI -22 to -5), p=0.005. E2 increased total fat mass, MAD 1007g (95%CI 124-1891), but not significantly, p=0.09. Android fat increased, MAD 164g (95%CI 41-286), p=0.04.

Conclusion: E2 did not change distal tibia vBMD in men undergoing ADT, despite decreasing bone remodelling and increasing regional aBMD and cortical vBMD. Contrary to our hypothesis, we provide suggestive evidence that E2 acting in the absence of testosterone, may increase total and regional fat mass in men.

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Increasing the detection of primary aldosteronism: a prospective study in Australian primary care

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Objective: To identify primary aldosteronism (PA) in newly diagnosed, treatment-naïve, hypertensive patients in primary care.

Design: Prospective study conducted in 2017-2020.

Setting: General Practitioners (GPs) from multiple practices across Melbourne (Victoria) were invited to screen their patients for PA at the time of the diagnosis of hypertension. Screening for PA was performed by measuring the aldosterone-to-renin ratio (ARR). Those with ARR ≥70 pmol/mU underwent the saline suppression test in a specialist referral centre to confirm the diagnosis of PA.

Participants: 247 primary care adults with blood pressure >140/90 mmHg on two or more occasions and not taking antihypertensive medications.

Main outcome measures: The diagnostic rate of PA, calculated as the percentage of patients with confirmed PA divided by the number of hypertensive patients screened for PA.

Results: Among the 247 participants, 62 (25%) had a positive screening test result and 35 (14% of all the participants; 95% confidence interval 10% to 19%) were confirmed to have PA. None of the patient characteristics (age, sex, blood pressure or serum potassium) distinguished the PA from the non-PA group.

Conclusion: PA was diagnosed in 14% of patients with newly diagnosed hypertension in primary care and GPs have an important role in actively screening for this specifically treatable cause of hypertension.

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Reproductive and metabolic health of young men conceived using ICSI

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Background:

Since its introduction for male infertility, the use of intracytoplasmic sperm injection (ICSI) has increased. Concerns include the heritability of infertility, effects of poor-quality spermatozoa on offspring health and epigenetic effects of the ICSI procedure. The long-term health of ICSI-conceived offspring is unclear.

Aim:

Compare reproductive and metabolic health of ICSI-conceived men (aged 18-25 years) to IVF-conceived and spontaneously-conceived (SC) controls.

Method:

This study is part of a project investigating health outcomes and epigenetic profiles of ICSI-conceived men. Age-matched controls were sourced from prior studies. Semen parameters and serum reproductive hormones were compared between 120 ICSI-conceived men and 356 SC controls. Resting blood pressure (BP), BMI, body surface area, and serum metabolic markers were compared between 121 ICSI-conceived, 74 IVF-conceived and 688 SC men.

Results:

Compared with SC controls, ICSI-conceived men had similar sperm output and total motile sperm count but lower mean total (55.3 vs 60.6%, p=0.003) and progressive (44.7 vs 53.9%, p<0.001) sperm motility with higher mean normal sperm morphology (8.5 vs 5.4%, p<0.001). Differences in progressive motility (ß -9.9, 95% CI -16.7- -3.0, p=0.01) and normal morphology (ß 4.3, 95% CI 3.0-5.7, p<0.001) remained significant after adjusting for confounders. ICSI-conceived men were no more likely to have below reference semen parameters. Semen parameters between ICSI fathers and sons were not correlated. Serum reproductive hormones were better in ICSI-conceived men.

ICSI-conceived men compared with SC controls had higher resting diastolic BP and higher homeostasis model assessment for insulin resistance (HOMA-IR), but a similar proportion had insulin resistance. Metabolic parameters of ICSI-conceived men and IVF-conceived controls were similar.

Conclusion:

This study is the largest to date and suggests comparable reproductive health of ICSI-conceived men to a population-representative cohort of SC men. Few metabolic differences of unclear significance were observed between ICSI-conceived men, and IVF-conceived and SC controls.

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Type 2 diabetes, fracture risk and mortality in two longitudinal population-based studies: Dubbo Osteoporosis Epidemiology Study (DOES) and the Canadian Multicentre osteoporosis study (CaM*os*)

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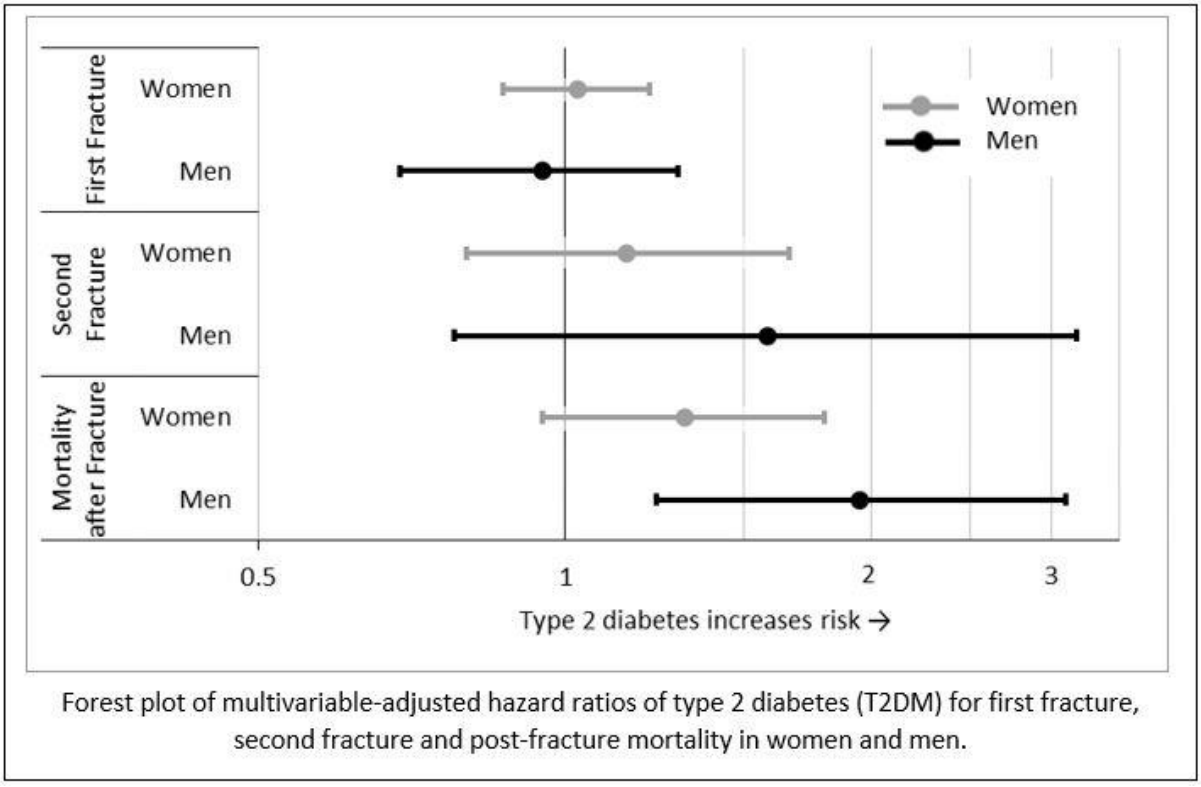
Type 2 diabetes (T2DM), osteoporosis and fracture are increasingly prevalent. Effect of T2DM on fractures and post-fracture outcomes remains unclear. This study examined the effect of T2DM on initial fracture, subsequent fracture and post-fracture mortality risk in elderly individuals in a combined cohort of the Dubbo Osteoporosis Epidemiological Study (DOES) and Canadian Multicentre Osteoporosis study (CaM*os*).

The study followed 9275 community-dwelling adults (6363 women) aged 60+ years over 13 years (IQR:7-15.1). DOES had fewer participants (39%) but longer follow-up (14 vs 11 years) compared to CaM*os*. Fractures were radiologically verified. Baseline and incident self-reported T2DM status was confirmed with medication use where available. We analysed T2DM as a time-dependent predictor using Cox's proportional hazards regression in gender-specific models with adjustment for known confounding factors. First fracture, subsequent fracture and post-fracture mortality were compared between those with and without diabetes.

T2DM was present in 471/2912 (16%) men and 782/6363 (12%) women (including incident T2DM in 159 men and 289 women). Fracture incidence was lower in both men and women with T2DM than those without (11% vs 15% men; 24% vs 29% women, respectively). Femoral neck bone mineral density (FNBMD) was significantly higher in women with T2DM compared to those without T2DM ($p < 0.01$). T2DM was not associated with increased risk of first or subsequent fracture in age-adjusted or after accounting for all potential confounding effects in either women or men (Figure). However, T2DM was associated with increased risk of post-fracture mortality in men (HR 1.95; 95%CI:1.23-3.11) but not in women (1.32; 0.95-1.81).

Women and men with T2DM have similar fracture rates to those without T2DM. After a fracture, mortality in women is similar between T2DM and non-diabetes subjects whereas mortality in men is further increased by T2DM. Fracture risk reduction is therefore important in men and women with T2DM.



Forest plot of multivariable-adjusted hazard ratios of type 2 diabetes (T2DM) for first fracture, second fracture and post-fracture mortality in women and men.

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Estradiol preserves endocortical and trabecular bone in an adolescent male-to-female mouse model of gender affirming hormone therapy.

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Elucidating the genetic effect of vitamin D on mesenchymal stem cells differentiation and function in vitro

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The active form of vitamin D (1, Alpha 25-Hydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$]) has been associated with multiple cellular processes, including bone formation. $1\alpha,25(OH)_2D$ deficiency results in the failure of bone formation leading to significant growth retardation and severe osteopenia. The anabolic effect of $1\alpha,25\text{-}(OH)_2D_3$ on bone could be explained via their action on differentiated mesenchymal stem cells (MSCs) either by facilitating osteoblastogenesis or inhibiting adipogenesis. In this study, we investigated the effect of $1\alpha,25\text{-}(OH)_2D_3$ on osteogenic and adipogenic differentiation of hMSCs treated with $1\alpha,25\text{-}(OH)_2D_3$ (10^{-8} mol) for 21 days. Treated and untreated undifferentiated MSCs were used as controls. We examined 12,000 human genes and expressed sequences tags on the array Human Genome U95A via Affymetrix DNA array. RT PCR confirmed those genes with higher and lower expression. We found that, compared to undifferentiating MSCs, differentiating cells treated with

1α,25-$(OH)_2D_3$ exhibited a significantly higher expression (more than two-fold change significance) of distinct osteogenic genes (e.g. *CYP24A1, AI131030, ITGAV, GYS1 and TBCD*) and adipogenic genes (e.g. *APOE, FBLN2, SYN1, G0S2, PARRES2 and CYP24A1*).

In addition, two genes (*FBLN2* and *G0S2)* showed significantly high expression in adipogenic conditions while decreased in the osteogenic conditions treated with 1α,25-$(OH)_2D_3$. Meanwhile, *AYP24A1*, associated with 1α,25-$(OH)_2D_3$ degradation, has reduced expression in adipogenic conditions compared to osteogenic and general conditions. In summary, our gene array analyses identified a direct effect of 1α,25-$(OH)_2D_3$ on a set of genes required in MSCs differentiation, thus improving our understanding of the effect of the active form on vitamin D on bone metabolism.

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Bone Microarchitecture and Estimated Failure Load are Deteriorated Whether Patients with Chronic Kidney Disease have Normal Bone Mineral Density, Osteopenia or Osteoporosis

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Introduction Measurement of bone mineral density (BMD) is recommended in patients with chronic kidney disease (CKD). However, most persons in the community and most patients with CKD have osteopenia, suggesting fracture risk is low. Bone loss compromises bone microarchitecture which increases fragility disproportionate to modest deficits in BMD. We therefore hypothesized that patients with CKD have reduced estimated failure load due to deterioration in microarchitecture irrespective of whether they have normal femoral neck (FN) BMD, osteopenia or osteoporosis.

Methods We measured distal tibial and distal radial microarchitecture in 128 patients with CKD and 275 age- and sex-matched controls using high resolution peripheral quantitative computed tomography, FN-BMD using bone densitometry and estimated failure load at the distal appendicular sites using finite element analysis.

Results Patients versus controls respectively had: lower tibial cortical area 219(40.7) vs. 237(35.3) mm^2, p =0.002, lower cortical volumetric BMD 543(80.7) *vs.* 642(81.7) mgHA/cm^3 due to higher porosity 69.6(6.19)*vs*. 61.9(6.48)% and lower matrix mineral density 64.2(0.62) *vs.* 65.1 (1.28)%, lower trabecular vBMD 92.2(41.1) *vs.* 149(43.0) mgHA/cm^3 due to fewer and spatially disrupted trabeculae, lower FN-BMD 0.78 (0.12) *vs.* 0.94 (0.14) g/cm^2 and reduced estimated failure load 3825(1152) *vs.* 5778(1467) N, all p<0.001. Deterioration in microarchitecture and estimated failure load was most severe in patients and controls with osteoporosis. Patients with CKD with osteopenia and normal FN-BMD had more deteriorated tibial microarchitecture and estimated failure load than controls with BMD in the same category. In univariate analyses, microarchitecture and FN-BMD were both associated with estimated failure load. In multivariable analyses, only microarchitecture was independently associated with estimated failure load and accounted for 87% of the variance.

Conclusions Bone fragility is likely to be present in patients with CKD despite them having osteopenia or normal BMD. Measuring microarchitecture may assist in targeting therapy to those at risk of fracture.

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Osteocalcin, Muscle Function and 15-year Falls-related Hospitalizations in Older Women: The Perth Longitudinal Study of Ageing Women

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Purpose: Undercarboxylated osteocalcin (ucOC) is suggested to be involved in muscle mass maintenance and strength, at least in animal models. In humans, the ucOC to total osteocalcin (tOC) ratio may be related to muscle function, a term combining muscle strength and physical function, and possibly falls risk, but the data is limited. We tested the hypothesis that ucOC and ucOC/tOC ratio are associated with muscle function and 15-year falls-related hospitalisations in older women.

Methods: Serum OC and ucOC was assessed in 1261 older women (mean age 75.2 ± 2.7 years) at year-1 of the Calcium Intake Fracture Outcome Study trial, forming the Perth Longitudinal Study of Ageing Women (PLSAW, 1998 to 2013). Timed-up-and-go (TUG) and grip strength was assessed at baseline (1998) and at 5 years. Falls-related hospitalisations over a 14.5-year follow-up was captured by the Hospital Morbidity Data Collection, via the Western Australian Data Linkage System.

Results: At baseline, women with higher ucOC/tOC ratio (quartile 4) had slower TUG performance compared to quartile 1 by 0.68 secs (~0.68 secs, p<0.01); grip strength and 5-year change in TUG and grip was not significantly different (p>0.05). Higher ucOC/tOC ratio was significantly associated with poorer TUG performance at baseline and 5-year change in performance (all p<0.05). Those with the highest ucOC/tOC had greater falls-related hospitalisations (unadjusted log rank p=0.004) that remained significant after adjusting for key variables (HR 1.31, 95% CI 1.09-1.57, p=0.004).

Conclusions: We identified many older women with high ucOC/tOC ratio that also have poorer physical function, including its long-term decline and increased risk of falls-related hospitalisation. This data supports the concept that quantifying ucOC/tOC ratio could be used as a predictor of these adverse outcomes, possibly enabling early intervention and minimising future fall risk. This should be explored in future.

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Fracture risk and use of angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers

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Background

Medications used to treat hypertension may affect fracture risk. This study investigated fracture risk for users of angiotensin converting enzyme inhibitors (ACEI) or angiotensin II receptor blockers (ARB).

Methods

Participants (899 men, 574 women, age≥50yr) were from the Geelong Osteoporosis Study. Medication use was self-reported and incident fractures were ascertained using radiological reports. Bone mineral density (BMD) was measured at the femoral neck. Participants were divided into four groups: 1) non-users without hypertension, 2) non-users with hypertension, 3) ACEI users and 4) ARB users. Dosage was calculated using the defined daily dose (DDD) criteria. Participants were followed from date of visit to first fracture, death or 31 December 2016, whichever occurred first. Cox proportional hazards models were used for analyses.

Results

At least one incident fracture was sustained by 156 men and 135 women over a median(IQR) of 11.5(6.2-13.2) and 10.9(6.3-11.6) years of follow-up, respectively.

For men, in unadjusted analyses, compared to non-users without hypertension, all three other groups had higher risk of fracture (Hazard Ratio (HR)(95%CI): 1.54(1.00-2.37), p=0.049; 1.90(1.18-3.05), p=0.008; 2.15(1.26-3.66), p=0.005, for non-users with hypertension, ACEI and ARB users respectively). Following adjustment for age, prior fracture and BMD, these associations became non-significant. A dose effect for ARB use was observed; men using lower doses (DDD≤1.0) had a higher risk of fracture than non-users without hypertension, in both unadjusted (2.66(1.34-5.29), p=0.005) and adjusted (2.03(1.01-4.10), p=0.047) analyses.

For women, unadjusted analyses showed a higher risk for ACEI users compared to non-users without hypertension (1.74(1.07-2.83), p=0.025). This was explained after adjustment for age, alcohol consumption, prior fracture and BMD (1.28(0.74-2.22), p=0.375). No other differences were observed.

Conclusion

ACEI or ARB use was not associated with increased risk of incident fracture in women. However, in men, lower dose ARB use was associated with an increased risk of fracture.

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Proteomics of secretory lysosomes unmasks new regulators of osteoclast function and skeletal bone mass

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Osteoclasts are central regulators of skeletal bone mass and are the only cell type capable of digesting bone. To achieve this, osteoclasts have evolved specialized lysosome-related organelles (LROs) termed 'secretory lysosomes' that give rise to the ruffled border upon synchronous fusion with the bone-apposed plasma membrane. Fusion of secretory lysosomes with the ruffled border equips its membrane with sets of nanoscale machinery that are requisite for extracellular acidification and bone digestion. Yet, despite their obvious importance in osteoclast function and thus fertile ground for the discovery of new anti-resorptive drug targets, our understanding of the molecular anatomy of the osteoclast secretory lysosome remains limited. In particular, we still lack elementary information regarding the nature and number of membrane proteins that define secretory lysosome identity and function.

Here, to expand the molecular inventory of membrane proteins operating on osteoclast secretory lysosomes, we have combined biochemical organelle enrichment methods with high-resolution tandem mass spectrometry (nLC-ESI-MS/MS) to unbiasedly survey the osteoclast secretory lysosome membrane proteome.

Using this approach, we unambiguously identified 4153 unique proteins. Of these, 181 integral membrane proteins were functionally assigned as membrane transport proteins (transportome) and 390 as regulators of membrane trafficking (traffickome). Stratification of the 'transportome' and 'traffickome' for proteins that: (i) are significantly enriched on secretory lysosome membranes (LogFC >1.5 and $p< 0.05$) and (ii) whose corresponding human orthologue lead SNPs reached genome-wide significance ($p< 5 \times 10^{-8}$) when cross-referenced against the largest genome-wide association study for a bone structural trait performed to date (eBMD, UKBioBank) established high-confidence lists of membrane transporters and trafficking proteins. By combining high-throughput siRNA-mediated gene knockdown studies in osteoclasts together with high-resolution confocal microscopy and novel genetic mouse models we demonstrate the power and utility of our approach to unmask new and physiologically relevant regulators of osteoclast function and skeletal bone mass.

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Characterising human skeletal stem cell populations in different skeletal compartments

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Human skeletal stem cells (SSCs) have been recently isolated from fetal long bones, however, the identity of adult SSCs remains elusive. Tissue-resident stem/progenitor cells support skeletal homeostasis and regeneration throughout life. Periosteum is a major source of cells involved in fracture healing. We hypothesize that progenitor populations are vary in different skeletal tissue compartments, and periosteum is enriched for SSC populations.

Cell surface marker expression was compared in human skeletal tissues by multi-colour flow cytometry with a 21-colour-panel. In brief, cells were isolated by enzymatic digestion of tissue from femoral heads of adults undergoing hip replacement for osteoarthritis: bone marrow, endosteum (or bone-associated cells) from cleaned trabecular bone, periosteum from the femoral neck, chondrocytes from the damaged and undamaged articular cartilage. Total haematopoietic cells were excluded using CD235a/CD15/CD31/CD45.

Within total non-haematopoietic cells, principal component analysis (PCA, Figure 1) of cell surface marker expression separates periosteum, cartilage and bone marrow/endosteum groups indicating substantial difference in marker expression in different tissues. Specifically, CD90, CD73, CD51, and CD34 were highly expressed in the periosteum. A number of markers were enriched in the cartilage, including CD105, CD106, CD133, CD164, CD271, PDGFRa, and PDPN, while CD24 was unique to the endosteum and bone marrow.

When total non-haematopoietic cells were sorted, surprisingly cartilage formed the most fibroblastic colonies (CFU-F), although they tended to remain smaller than periosteum and endosteum-derived CFU-F. Bone marrow did not form any colonies at the density tested. CD73+ and PDPN+ cells from periosteum, cartilage, and endosteum formed more CFU-F compared with total non-haematopoietic cells, and all these populations were highly osteogenic in vitro.

Using cell surface markers we identified populations of skeletal stem/progenitor cells with osteogenic potential in vitro. Our findings suggest that progenitor populations vary in different skeletal compartments and there may be cell populations unique to certain tissue compartments.

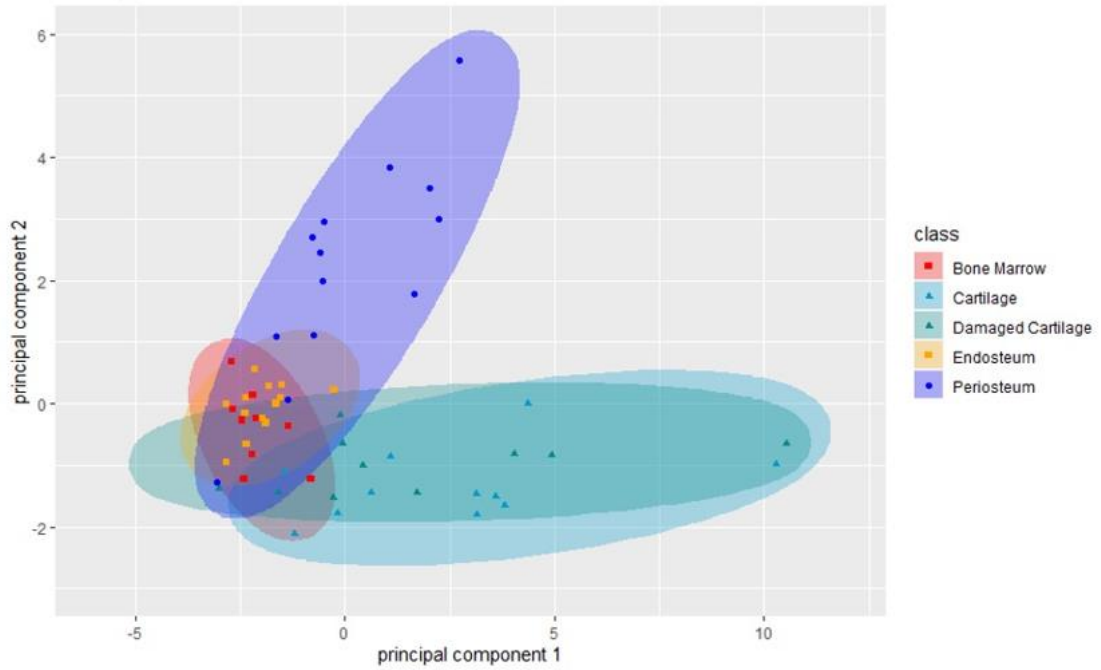

Figure 1. Principal component analysis visualisation of difference in surface marker expression of a set of 16 putative stem/progenitor cell surface markers in different skeletal tissues.

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Endothelial protein C receptor (EPCR) is deranged in preeclampsia

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Preeclampsia impacts 2-8% of pregnancies and is characterised by placental and endothelial dysfunction(1). Endothelial Protein C receptor (EPCR) is localised to endothelial cells and abundantly expressed in placental trophoblasts(2). This study aimed to characterise EPCR in preeclampsia.

EPCR was measured in placentas from patients with preterm preeclampsia and both its mRNA and protein were significantly ($p<0.0001$) increased. Plasma EPCR and its ligand, Protein C (PROC) were then assessed (n=46 preeclampsia vs n=16 controls). Plasma EPCR was increased ($p=0.0099$), while PROC was decreased ($p=0.0083$) in preeclamptic patients. In contrast, circulating EPCR at 36 weeks' gestation was not different in asymptomatic patients destined to develop preeclampsia at term (n=23 who later developed preeclampsia vs n=181 controls).

Given preeclampsia is associated with placental hypoxia and inflammation, primary trophoblasts were exposed to hypoxia or the pro-inflammatory cytokine TNF-α. EPCR mRNA expression was increased following exposure to hypoxia ($p=0.0079$), whilst TNF-α dose dependently decreased ($p=0.0005$) EPCR. Metformin is a potential therapeutic for preeclampsia(3), thus we next assessed its effect on EPCR in isolated primary trophoblasts. Interestingly, metformin reduced EPCR expression. Finally, to assess whether circulating EPCR might also be sourced from dysfunctional maternal endothelium, primary endothelial cells (isolated from human umbilical vein endothelial cells) were treated with TNF-α, and endothelial dysfunction markers and EPCR assessed. Exposure to TNF-α induced dysfunction (elevated VCAM-1, ET-1), but significantly decreased EPCR expression ($p=0.0079$).

In conclusion, EPCR is increased in preterm preeclampsia. Placental hypoxia possibly contributes to this elevation and levels can be reduced by preeclampsia therapeutic metformin. Although EPCR is elevated in established disease, plasma EPCR is not altered preceding term diagnosis, therefore it is not a useful predictive biomarker. Further study will aid in elucidating the potential contribution of elevated placental EPCR to the pathogenesis of preeclampsia and determine its potential as a novel therapeutic target.

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Alterations in placental endothelial and perivascular heterogeneity could contribute to the pathophysiology of fetal growth restriction

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Placentae from FGR pregnancies are smaller, with a reduced vascular network that impairs placental exchange. However, little is known about the complex differentiation events and distinct cell types that underpin normal and abnormal placental vascular formation/function. We aimed to characterise the vascular and perivascular cell populations within the placental villous core across gestation, and determine whether differences exist in FGR placentae.

First-trimester (7-12 weeks, n=6), normal term (37-40 weeks, n=3), or FGR (<5th percentile, 37-40 weeks, n=3) placentae were denuded of trophoblast and enzymatically digested. The resulting cells were phenotyped using 23-colour flow cytometry. First-trimester (<9 weeks) endothelial cells (CD31^{+}CD34^{+}) were FACS sorted (n=3) and cultured in endothelial differentiation (EGM-2+VEGF165 on Matrigel) or smooth muscle differentiation (advanced-DMEM/F12+TGF-β1) conditions. Differentiation was assessed by multi-colour flow cytometry and immunohistochemistry.

Endothelial and perivascular cell phenotypes and heterogeneity differed across gestation. Proportionally, in FGR placentae endothelial cells constituted 3-fold fewer total villous core cells ($p<0.05$), contributing to an increased perivascular:endothelial cell ratio (2.6-fold higher, $p<0.05$). Term endothelial cells expressed “mature” endothelial markers (CD36^{+}CD146^{+}), whilst first-trimester demonstrated an “immature” phenotype (CD144$^{+/low}$CD36^{-}CD146^{low}). Sub-populations of endothelial cells co-expressed mesenchymal markers (CD90, CD26) suggesting a potential to undergo Endothelial to Mesenchymal Transition (EndMT). This EndMT was confirmed *in vitro*, where first trimester placental endothelial cells lost endothelial (CD31, CD34, CD144) and gained mesenchymal (CD90, CD26) marker expression even in endothelial differentiation conditions. In smooth muscle differentiation conditions, endothelial cells expressed α-SMA and calponin, showing further progression towards a contractile phenotype.

Differences in villous core cell heterogeneity over gestation reflect different stages of vascular development and organ growth. This work suggests that dysregulated EndMT could contribute to the increased perivascular:endothelial cell ratios in FGR, and in turn the increased vascular resistance seen in this pathology

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Regulatory T cells modulate uterine natural killer cells to promote spiral artery remodelling in mice

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Early-onset preeclampsia is preceded by abnormal placentation and impaired spiral artery remodelling, and often results in fetal growth restriction. Foxp3^{+} regulatory T (Treg) cells, a specialised T cell subset with key roles in modulating inflammation, are deficient in many women with preeclampsia. Previously we showed, using acute transient depletion of Treg cells in early pregnancy elicited by diphtheria toxin (DT) administration to transgenic *Foxp3-DTR* mice, that Treg cells are critical for normal spiral artery remodelling and act to reduce the resistance index of the uterine artery in mid-gestation. Uterine natural killer (uNK) cells are also implicated in mediating decidual spiral artery remodelling, but whether Treg-uNK cells interactions are important for vascular remodelling is unknown. We therefore hypothesised that impaired spiral artery remodelling after Treg depletion is accompanied by altered uNK cell parameters. Mated *Foxp3*-DTR mice were injected with DT on gestational day 3.5 and 5.5 to deplete Treg cells and vehicle-treated *Foxp3-DTR* mice served as controls. On gestational day 10.5 uNK cell abundance was assessed by histochemical analysis and flow cytometry. Decidual sections stained with Dolichos Biflorus Agglutinin (DBA) lectin, which reacts with uNK cells, revealed a 20% decline in uNK abundance($P<0.001$). Two distinct subsets of uNK cells have been described – tissue-resident and conventional (circulating) – each with distinct roles in angiogenesis and tissue remodelling. Flow cytometry was performed to further characterise the uNK decline using NK-cell associated markers, and the tissue residency marker CD49a to distinguish tissue-resident and conventional uNK subsets. Preliminary data revealed a decrease in conventional NK1.1^{+}Nkp46^{+}EOMES^{+}CD49a^{-} uNK cells within the uterus and decidua at mid-gestation following Treg depletion($P<0.05$). These data suggest that Treg cells facilitate spiral artery remodelling through regulating conventional uNK cell abundance in the uterus and decidua. It will therefore be important to investigate decidual Treg cell-uNK cell interactions in women with preeclampsia.

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A potential role for the (pro)renin receptor and soluble (pro)renin receptor in the pathogenesis of choriocarcinoma

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Background/Aims: Choriocarcinoma is a trophoblastic tumour, most highly prevalent in Asian populations. The (pro)renin receptor ((P)RR) stimulates signalling pathways that enhance cancer progression (invasion, proliferation, and angiogenesis). We aimed to determine if (P)RR is involved in choriocarcinoma pathogenesis. To do this we used the BeWo choriocarcinoma cell line, which comprises both cytotrophoblast (mononuclear) and syncytiotrophoblast (multinuclear) cells. BeWo cells can be induced to syncytialise with forskolin. We aimed to determine which subset of these cells are more invasive and whether (P)RR affects the ability of BeWo cells to syncytialise and/or invade. Additionally, we aimed to determine if soluble (P)RR (s(P)RR) could be a biomarker for choriocarcinoma invasiveness and how it is cleaved from full-length (P)RR in BeWo cells.

Methods: BeWo choriocarcinoma cells were treated with (P)RR siRNA, Furin siRNA, negative control siRNA, DEC-RVKR-CMK (a furin enzyme inhibitor), or PF 429242 (a site 1 protease inhibitor), before treatment with forskolin (to induce syncytialisation) or vehicle (DMSO). Choriocarcinoma cell invasion was measured using the xCELLigence Real-time Cell Analysis system. Syncytialisation was measured by assessing E-cadherin (immunoblot/immunocytochemistry) and hCG secretion (ELISA). Soluble (P)RR levels were measured by ELISA.

Results: Forskolin successfully induced syncytialisation and significantly increased choriocarcinoma cell invasion ($P<0.0001$). (P)RR siRNA inhibited both syncytialisation (as evidenced by decreased hCG secretion ($P=0.005$) and the percent of nuclei in syncytia ($P=0.05$)) and invasion ($P=0.046$). Additionally, forskolin increased s(P)RR levels ($P=0.02$). (P)RR siRNA, Furin siRNA and DEC-RVKR-CMK significantly reduced s(P)RR secretion (all $P<0.0001$), PF 429242 had no effect.

Conclusions: The syncytiotrophoblast in choriocarcinoma is invasive and (P)RR knockdown decreases forskolin-induced syncytialisation and invasion. Hence, (P)RR could play a role in the pathogenesis of choriocarcinoma. Since s(P)RR levels reflect syncytialisation, invasion and (P)RR levels, we hypothesise that s(P)RR could be a marker of choriocarcinoma cell invasion. Furin, not site 1 protease, cleaves s(P)RR in choriocarcinoma cells.

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Malate dehydrogenase 1B is a testis enriched energy protein required for sperm function and male fertility

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Publish consent withheld

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Field fertility of a novel bovine sperm storage medium following seven days of ambient temperature storage

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Northern Australian beef producers have been slow to adopt artificial insemination (AI) practices, in favour of the more traditional hands-off approach of 'natural' breeding. However, the rising demand for global food security requires an increased rate of genetic gain for production traits, which can only be met using AI. By simplifying the logistics around AI and improving pregnancy rates, we aim to make this a more feasible option for beef producers. To do this we developed a novel medium, SpermSafe-B (SS-B), containing alternative osmolytes, additional energy sources, and antioxidant supplementation capable of supporting sperm metabolism during ambient temperature storage. After thorough sperm parameter assessments and *in vitro* fertilisation trials, the field fertility of bull spermatozoa stored in SS-B was evaluated. Semen was collected via electroejaculation from three bulls with high quality spermatozoa selected using a 45/90 discontinuous BoviPure™ density gradient, resuspended at a concentration of ~50 million/mL in SS-B, and stored in the dark at ambient temperature (~22°C). No significant decline in total (TM) or progressive (PM) motility was recorded after seven days of storage (TM: 83.3±3.06% vs. 73.0±3.18%; PM: 82.8±2.83% vs. 70.9±3.97%; at Day 0 and Day 7 respectively; $P>0.15$). At Day 7, fixed-time AI following oestrus synchronisation was performed on 18 2-year-old virgin heifers using an insemination dose of 0.5 mL (15.75-18.75 million progressively motile spermatozoa). Ultrasonographic pregnancy diagnosis at 33 days post-insemination confirmed 14 of 18 (77.8%) heifers were in calf. This is the first trial of its kind assessing the field fertility of bull spermatozoa in an ambient temperature sperm storage medium. While this focused trial did not directly compare to current frozen-thawed AI protocols, the pregnancy rate achieved using spermatozoa stored in SS-B was higher than the industry-standard using cryopreserved semen (60%). These results serve as proof of concept to commence a large-scale fertility trial.

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Fabrication on the microscale: two-photon polymerised device for microinjection

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Intracytoplasmic sperm injection (ICSI) addresses male sub-fertility by injecting a spermatozoon into the oocyte to induce fertilisation. It is a technically challenging procedure that requires dual micromanipulation. In the IVF clinic, only highly skilled embryologists perform ICSI. However, variability in success exists between embryologists within and across clinics. Poor success may be the result of the procedure causing mechanical stress on the oocyte, leading to impaired fertilisation and embryo development. We hypothesise that minimising oocyte handling during ICSI will simplify the procedure and thus improve embryo production. To address this, we designed and fabricated a micrometre-scale transparent device to house the oocyte. The device avoids the use of a holding pipette, requiring only one micromanipulator to perform microinjection of sperm. Computer aided design software was used to design the device, which consisted of 2 components, *Pod* (670 x 235 x 353 µm; l x w x h) and *Garage* (1150 x 450 x 345 µm). The fabrication of the device was performed by the optical method of 2-photon polymerisation and houses individual oocytes within a single *Pod*. We show that an array of *Pods* docked within a *Garage* enabled high-throughput microinjection of multiple oocytes. Importantly, the *Pod/Garage* is embryo safe, demonstrated by the gold-standard mouse embryo assay. The use of the *Pod/Garage* array for ICSI enabled rapid tracing of oocytes that had already undergone, vs. those that still required microinjection. Finally, we demonstrate that subsequent embryo development can be carried out in the same device with development to blastocyst not significantly different to standard microdrop culture (87.4% ± 3.0% *vs* 85.3% ± 7.1%, respectively, $P > 0.05$). This work could improve embryo production by removing intra-operator variability and may form a precursor to automated ICSI.

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Cripto promotes pluripotency and malignancy of the male germline in mice

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Contrary to their somatic counterparts, primordial germ cells (PGCs) maintain an active pluripotency network and can give rise to any cell types. Following migration to the developing male gonad, the PGC pluripotency program is progressively shut down as they commit to the pro-spermatogonia fate during a short developmental window (E11.5 to E14.5). PGCs that fail to differentiate, by maintaining pluripotency, harbour malignant potential and are considered the origin of testicular germ cell tumours in humans.

We aimed to explore the role of *Cripto* in this developmental process. Cripto is a co-receptor for Nodal signalling and is initially expressed in PGCs until E11.5, when its expression decreases as PGCs lose pluripotency. Because we found that CRIPTO is overexpressed in pluripotent human testicular germ cell tumours (incl. seminomas, embryonic carcinoma), we hypothesised that maintenance of Cripto expression in PGCs might drive malignant transformation.

We generated a novel, germ cell specific, CRE recombinase mouse line (*Ddx4-iCre*) to over-express *Cripto* in PGCs from E11.5. We found that Cripto overexpression in PGCs leads to the maintenance of a pluripotent state. Furthermore, Cripto-overexpressing (Cripto-OE) PGCs failed to correctly differentiate, leading to germ cell depletion by the time of birth. Although the Cripto-OE^{PGC} model held great potential to replicate the human pathology of testicular germ cell tumours, the complete lack of germ cells in Cripto-OE^{PGC} adult mice hindered our ability to investigate whether testicular tumours would form. To circumvent the germ cell loss, we placed our Cripto-OE model on a BAX-KO background (Bax is a pro-apoptotic gene). Here, as early as 8 weeks of age, we observed Cripto-OE germ cells persisting in homogenous clusters that morphologically resemble human intra-tubular seminomas.

Altogether, we propose an oncogenic role for Cripto in the context of PGC development and present a possible novel mouse model of seminoma germ cell tumours.

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An *ex vivo* approach to understanding sex-specific differences in placental androgen signalling in the presence and absence of inflammation

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Background: The mechanisms that contribute to continued male intrauterine growth in response to an adverse maternal environment, such as those brought on by maternal asthma, remain largely undefined but may, in part, be mediated by androgen-mediated signalling. We previously reported the expression of multiple AR protein isoforms in the human placenta vary in

response to maternal asthma and proposed the novel AR-45 isoform to be integral in mediating male-specific androgen-dependent placental signalling. This was recently supported by work in a sheep model of maternal allergic asthma that identified AR-45 activity to be increased; however, we were unable to examine the effect of sex. Thus, in the current study we have utilised an *ex vivo* approach to understand sex-specific differences in placental androgen signalling in the presence and absence of inflammation using human term villous placental explants.

Methods: Explants (2mm sections; approx. 100mg wet weight) were cultured in the presence and absence of 0.1nM dihydrotestosterone (DHT), 1µg/ml lipopolysaccharide (LPS), or DHT+LPS for 24hr. Tissue was used for gene expression and subcellular AR protein isoform expression.

Results: Cytoplasmic and nuclear AR protein isoforms expression did not vary between culture conditions in either sex. AR-45 fold-change expression (normalised to untreated samples) was significantly increased in male placentae in the presence of DHT when compared with females. The activity of AR-45 was upregulated in male placentae cultured in DHT, LPS and DHT+LPS, but this was not observed in females. There were no changes in the expression of androgen-mediated downstream targets in males in response to culture conditions, but females had significantly reduced *IGF1R* expression in response to LPS.

Conclusion: Increased AR-45 activity in the presence of inflammation with or without androgens may drive continued male feto-placental growth in an adverse maternal environment via maintained expression of downstream growth targets.

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Analysis of novel glucocorticoid receptor agonists as potential treatments for preterm birth

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Glucocorticoid (GC) signalling is essential for normal fetal lung development. During late gestation a surge of endogenous GCs rapidly matures the lung by thinning the mesenchymal tissue and driving an increase in gas exchange surface area. Currently in situations of imminent preterm birth potent synthetic GCs such as betamethasone or dexamethasone (Dex) are administered antenatally to accelerate fetal lung maturation and reduce the risk of respiratory distress syndrome. There are however growing concerns that systemic exposure to powerful synthetic glucocorticoids is associated with detrimental side effects, particularly in the developing fetal brain. We are currently assessing novel activatable and partial agonists of the glucocorticoid receptor (GR) as new potential antenatal steroid treatments of preterm birth. One such GR agonist is a steroid prodrug called ciclesonide (Cic) that is activated *in vivo* by a family of serine-esterase enzymes, called the carboxylesterases (Ces). In this study we have assessed the expression of Ces enzymes in fetal organs and also compared the effect of activated Cic to dexamethasone for the regulation of key GR-regulated respiratory genes. We show that one isoform Ces1d is highly expressed in the fetal mouse lung but is absent in the brain thereby reducing Cic activation in the mouse fetal brain. To assess Cic activity in the fetal lung, primary fetal mouse fibroblast cell cultures from WT and GR-null mice were treated with Dex, Cic and its active form des-Cic for 6 hours and changes in the fibroblast transcriptome assessed by RT-qPCR. Analysis of four known GR regulated genes, Fkbp5, Crispld2, Tgm2 and Zbtb16, showed that activated Cic strongly induced expression of these genes that was dependent on a functional GR. This preliminary data indicates that Cic regulates respiratory genes in a similar way to Dex but may have negligible activation in the fetal brain thereby sparing systemic side-effects.

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Does Capsaicin "Beige" Human White Fat that has been Transplanted into Mice and Exhibit Improvements in Mouse Metabolism?

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BACKGROUND: Obesity is a major health concern and increases risk of metabolic syndrome, type 2 diabetes, dyslipidemia, cardiovascular diseases and many cancers. Obesity occurs with decreased physical activity and increased caloric intake. Clinical management is still limited. Brown adipose tissue is a thermogenic organ which expresses uncoupling protein 1. When activated it increases energy expenditure by up to 20%. Recent evidence suggests that white adipose tissue can be 'browned' and have similar characteristics, called beige fat.

AIMS: This project tested a "beiging" agent capsaicin to determine whether it could brown human fat and improve metabolism.

METHODS: We used a "Humanised Mouse Model" where human fat is inserted intra-abdominally in immune suppressed mice (to avoid rejection). Mice were fed ad libitum normal or a high fat diet (45% calories from fat) ± 0.03% capsaicin. Metabolic studies were conducted before and after fat transplant and 12 weeks on diets. These studies included glucose and insulin tolerance tests and metabolic cages. mRNA and histology samples were taken.

RESULTS: Results show increases in energy expenditure (vCO_2 and vO_2) in mice fed capsaicin compared to their respective controls. High fat diet + capsaicin fed mice showed improvements in glucose tolerance and insulin sensitivity. The human fat showed up-regulation of uncoupling protein 1.

CONCULSONS: These results indicate beiging of human white fat is possible and has the potential to improve metabolism. A 20% increase in energy expenditure has the potential to cause clinically significant improvements in obesity. Further studies will examine combination therapies to optimise browning.

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Concurrent Betaine Administration Enhances the Benefits of Exercise in a Mouse Model of Diet-Induced Obesity

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Health concerns around obesity have driven the search for novel approaches to therapy including lifestyle changes and supplementation of diet. Previous work showed improvements in lipid metabolism in obese mice supplemented with betaine, and in healthy people, betaine has been used to enhance the benefits of exercise. Here we explored the potential synergy between betaine and exercise as an intervention for high-fat diet (HFD)-induced obesity. We hypothesized that combining treadmill exercise and orally administered betaine would provide synergistic benefits for ameliorating diet-induced obesity.

Male C57BL/6J mice were fed either chow (Con) or HFD to induce obesity then HFD mice were separated into these groups: HFD, HFD with betaine (HB), HFD with exercise (HEx), and HFD with betaine and exercise (HBEx); treadmill exercise (15 m/min for 45 min, 6 days/week) and/or 1.5% (w/v) betaine in drinking water. Body composition and intraperitoneal glucose tolerance tests (GTT) were assessed prior to and after treatment (5 weeks).

Administration of betaine alone did not affect glucose tolerance or insulin secretion during GTT. Exercise lowered body weight and fat composition, with or without betaine. Whilst HEx mice exhibited mildly improved glucose tolerance during GTT, HBEx mice had significantly reduced blood glucose levels compared to HFD mice, as well as reduced insulin levels, which were comparable to Con. Interestingly, all HFD-groups showed upregulation of hepatic *Pparg* despite reduced hepatic triglycerides in mice undergoing exercise. Moreover, HFD-induced increases in *Mpc1* and *Pc* were normalized in mice receiving combined exercise and betaine treatment, suggesting the possibility that the improvements observed during GTT may be linked to changes in glucose metabolism. Thus, 1.5% (w/v) betaine in drinking water augmented the beneficial effects of exercise on metabolic health in obese mice, which may be facilitated by processes involved in glucose metabolism with limited effects on hepatic lipid metabolism.

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Characterization of 11bhsd1l a novel member of the 11bhydroxysteroid dehydrogenase enzyme family

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The 11b-Hydroxysteroid dehydrogenases (11bHSD) are a subfamily of short-chain dehydrogenase/reductase enzymes, which regulate cortisol metabolism and actions in vivo. Although much is known about the biology and function of 11bHSD1 and 11bHSD2, little is known of a recently discovered member of the 11bHSD family, called 11bHSD1L which is expressed in primates, fish, plants, and other mammals, but is absent in rodents (1). Current expression profiling in human, sheep and marmoset tissues suggest that 11bHSD1L may be linked to the regulation of reproduction through the hypothalamic-pituitary-gonadal (HPG) axis or to an unknown regulatory role in the brain. The definitive physiological substrate for 11bHSD1L is unknown. We have studied the expression of 11bHSD1L in the non-human primate marmoset and show that 11bHSD1L mRNA levels were highest in regions of the brain and reproductive organs, which parallels expression patterns in human tissues. Analysis of two major 11bHSD1L RNA splice forms, derived from exon 8, identified that the 11bHSD1L 'Exon 8A' splice variant was the dominant isoform with mRNA levels 100 times that of the 'Exon 8B' isoform in the marmoset ovary and cortex of the brain. Localization of 11bHSD1L protein in marmoset tissue sections by immunohistochemistry identified several cell populations previously unreported for 11bHSD1L, that included Sertoli cells in the testis, and theca cells in the ovary - further implicating 11bHSD1L as a potential novel regulator of the HPG Axis. There is some evidence that 11bHSD1L is a weak oxidase of the steroid cortisol. Preliminary experiments using a dual-luciferase based cell transfection reporter system shows that cortisol is unlikely to be a substrate of 11bHSD1L, distinguishing it from other members of the 11bHSD subfamily of enzymes.

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Glucocorticoid regulation of primary ciliogenesis in the developing mouse kidney

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Glucocorticoid (GC) hormones have well characterised roles for the development of several fetal organs, however, specific developmental roles of GCs in the fetal kidney are relatively unknown. We have explored both the expression and localisation of the glucocorticoid receptor (GR) within developing kidney structures during organogenesis and the effect of eliminating GR expression using gene-targeted GR-null mice. We show that loss of the GR has a profound effect on the renal transcriptome with altered expression of 454 genes in the GR-null mouse kidney at E18.5 of gestation with more genes significantly downregulated (305) than upregulated (99) relative to wild-type controls, including the key primary cilia-associated gene Centrosomal protein 290 (*Cep290*) with a fold change of -3.6. Two renal tubule markers, Kidney androgen regulated protein (*Kap*) (proximal tubule marker) and S100 calcium binding protein G (*S100g*) (distal tubule marker) were also downregulated with fold changes of -8.61 and -2.5 respectively. We demonstrate that primary cilium length is significantly decreased in kidney proximal tubule cells in E18.5 GR-null mice (5.23 + 0.12, µm + SEM) compared with wild type controls (6.27+ 0.15, µm + SEM). Finally, we demonstrate that in the IMCD3 mouse collecting duct cell line dexamethasone treatment increases the length of primary cilia compared to vehicle controls (vehicle 2.49 + 0.03, µm + SEM vs dexamethasone 3.17 + 0.05 µm + SEM), an effect blocked by pre-treatment

with the GR antagonist RU486. Taken together these results indicate that GC signalling via the GR contributes to the formation of primary cilia in the proximal tubule and is required for normal kidney development.

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Oxidative stress promotes glucocorticoid-induced mineralocorticoid receptor (MR) transcriptional activity in cardiomyocytes

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Publish consent withheld

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Mitochondrial dysfunction aligns with risk for diabetic kidney disease in youth with type 1 diabetes

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Background

Growing evidence demonstrates that diabetic kidney disease (DKD) begins as early as adolescence, with the highest tertile of urinary albumin-to-creatinine ratio (uACR) being identified as those individuals at elevated risk. The best-pactise regimens used to combat DKD in adults have proven ineffective in Phase III clinical trials, indicating that in early T1D other factors may confer the risk of DKD.

This study tested the hypothesis that mitochondrial dysfunction in adolescents with T1D may contribute to an increased risk in developing DKD.

Methods

This cross-sectional cohort study recruited 100 young adults with T1D. Mean uACR was determined and the cohort was then divided into the low-risk group (uACR <1.16 mg/mmol; n=66) and the high-risk group for DKD and CVD (uACR ≥1.17; n=34).

Peripheral blood mononuclear cells (PBMCs) from all participants were isolated and their mitochondrial to nuclear DNA ratios were determined. A Seahorse mitochondrial stress test was also performed. Flow cytometry was used to quantify circulating T cells.

Primary proximal tubule epithelial cells (PTECs) were treated with the serum of the participants and the mitochondrial density and levels of SGLT2 and Collagen IV were measured using flow cytometry.

Results

PBMCs from the high-risk group had a higher oxygen consumption rate but lower basal respiration (P=0.01) and ATP-linked respiration (P=0.03). There was a negative correlation between the number of $CD3^+$ T cells and the PBMC mitochondrial to nuclear DNA ratio in both the low-risk group (slope = -3.5; P=0.045) and the high-risk group (slope = -5.2; P=0.0022). PTECS exposed to the plasma of high-risk patients had a trend towards increased mitochondria (P=0.08) and had statistically significant increases in Collogen IV (P=0.049) and SGLT2 levels (P=0.01).

Conclusion

Young adults with T1D at high risk for DKD and CVD have signs of changed mitochondrial function and possess altered proportions of circulating T cells.

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Production of bone matrix in recovery from bone loss during breast-feeding is independent of 1,25-dihydroxy-Vitamin D_3 (calcitriol)

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During breastfeeding, calcium for the infant is supplied by resorption of the maternal skeleton; this bone is restored during weaning, a process reported to be independent of calcitriol. However, that evidence was from 1-α-hydroxylase or Vitamin D receptor (VDR) null mice fed a calcium-enriched "rescue" diet to prevent rickets. Here we assess whether skeletal recovery from lactation-induced bone loss is calcitriol-independent on a normal diet.

Wild type and VDR null mice were fed the rescue diet from birth to end lactation and switched to a normal diet at weaning. Bones were collected at baseline, day 18.5 of pregnancy, 21 days of lactation, and 28 days post-weaning (recovery). Bones were also collected from non-mated mice of both genotypes given the same diets at the same ages; these showed no bone loss compared to controls.

Cortical thickness and maximal bone strength were both significantly reduced by 25% in lactating wild type mice compared to baseline and age-matched controls and were both completely restored post-weaning. In contrast, VDR null mice had a similar 25% reduction in cortical thickness and maximal strength with lactation, but neither were restored post-weaning. VDR null femora at 28 days post-weaning exhibited high cortical porosity and abundant very low-density bone on the endocortical surface.

Low-, mid-, and high-density measurements revealed that wild types lost and restored high-density bone. However, while VDR null mice lost high density bone in lactation, they formed only low-density bone post-weaning, to 3x normal levels. Histology revealed abnormally thick seams of unmineralised osteoid and significantly greater extent of osteoblast and osteoid on both endocortical and intracortical surfaces.

We conclude that calcitriol is required for recovery from lactation-induced bone loss on a normal diet, and that while bone matrix production during recovery is calcitriol-independent, it lacks mineral likely due to insufficient dietary calcium and phosphate supply.

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Bisphosphonate drugs have effects outside the skeleton and inhibit the mevalonate pathway in alveolar macrophages in lung

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Nitrogen-containing bisphosphonates (N-BPs) are well-established treatments for post-menopausal osteoporosis, metastatic bone disease and other skeletal disorders. These drugs inhibit bone resorption by blocking the mevalonate pathway in osteoclasts, thereby preventing protein prenylation. The high affinity of N-BPs for bone mineral confers bone-targeting and specificity for osteoclasts, hence, it's widely considered that N-BPs only act in the skeleton. However, accumulating epidemiological data suggest that N-BPs also have beneficial, pleiotropic effects outside bone, including decreased risk of mortality from pneumonia, but the mechanisms remain unknown.

Like osteoclasts, macrophages in culture and in tumours *in vivo* efficiently internalise N-BP by endocytosis. Here, we sought to identify whether N-BPs could also act on macrophages in the lung *in vivo*, and alter immune function. We used fluorescently-labelled N-BP and flow cytometry to detect drug uptake into cells. 24 hours after *iv* injection, we detected N-BP in >98% of $CD11b^{lo/-}CD11c^{hi}F4/80^{+}$ alveolar macrophages in lung, as well as 80% of peritoneal cells (almost entirely $CD11b^{+}F4/80^{+}$ peritoneal macrophages). Characteristic features of N-BP action – inhibition of protein prenylation and build-up of the metabolite isopentenyl pyrophosphate – were also detected in alveolar and peritoneal macrophages 48 hours after one *iv* dose of the N-BP zoledronate. Importantly, a single *iv* dose of zoledronate significantly enhanced 2.5-5-fold the release of proinflammatory cytokines and chemokines (including IL-1beta, IL-6, TNFalpha, CXCL1, CCL2,3,4,5) into bronchoalveolar lavage fluid after intranasal endotoxin stimulation.

In summary, we present new evidence that N-BP treatment targets tissue resident macrophages *in vivo* in lung and peritoneum and enhances immune responses, dispelling the long-held dogma that bisphosphonates act only in bone. Given that inhibition of the mevalonate pathway is known to have a variety of anti-microbial effects, we propose that the apparent beneficial effects of N-BPs on pneumonia infection and mortality occur via actions on alveolar macrophages in the lung.

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Osteoblast-derived egfl6 couples angiogenesis to osteogenesis during bone repair

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Introduction. Angiogenesis and osteogenesis are considered two indispensable and highly coupled steps involved in successful bone repair [1, 2]. However, the nature and identities of factors that regulate the coupling process remain largely elusive. We previously found that epidermal growth factor-like protein 6 (EGFL6) is an angiogenic factor that is specifically and distinctively up-regulated during osteoblast differentiation [3]. In contrast with most currently known osteoblast-derived coupling factors, EGFL6 is highlighted with its little or low expression in other cells and tissues. This study aims to uncover the role of EGFL6 in bone repair. **Methods.** Primary bone marrow mesenchymal stem cells (MSCs) and MC3T3-E1 were transduced with lentiviral silencing or overexpression constructs targeting EGFL6. Bone-related markers were examined by qPCR and Western Blot (WB) assay. EGFL6 global and osteoblast-specific knockout (KO) mice were established to examine the bone phenotype under

physiological conditions by using micro-CT scanning and bone histomorphometry analysis. Bone-specific type H vessels were identified using immunofluorescent staining of CD31 and Endomucin (EMCN). Mono-cortical bone defects were created in both wildtype (WT) and KO mice. **Results and discussion.** We found that overexpression of EGFL6 significantly enhances osteogenic capacity in vitro by augmenting bone morphogenic protein (BMP)-Smad and MAPK signalling, whereas downregulation of EGFL6 diminishes osteoblastic mineralization. Interestingly, osteoblast differentiation was not affected by the exogenous addition of EGFL6 protein, thereby indicating that EGFL6 may regulate osteoblastic function in an intracrine manner. Mice with osteoblast-specific and global knockout of EGFL6 surprisingly exhibit a normal bone phenotype under the physiological condition. However, EGFL6-deficiency leads to compromised bone repair in the bone defect model which is characterized by the decreased formation of type H vessels as well as osteoblast lineage cells. **Conclusions.** Taken together, our findings demonstrated that EGFL6 serves as an essential regulator to couple osteogenesis to angiogenesis during bone repair

1. 1. Kusumbe et al., Nature. 2014; 507: 323-8. 2. Ramasamy et al. Nature. 2014; 507: 376-80. 3. Chim et al., J Biol Chem. 2011; 286: 22035-46.

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Combination treatment with undercarboxylated osteocalcin and ibandronate protects against hind-limb immobilisation-induced muscle wasting

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Background Immobilisation leads to muscle wasting, which may cause insulin resistance. Undercarboxylated osteocalcin (ucOC) is suggested to improve muscle mass and glucose metabolism. Bisphosphonates were recently reported to protect against muscle wasting, independent of ucOC. We tested the hypothesis that combination treatment with ucOC and bisphosphonates exerts a superior protective effect against immobilisation-induced muscle wasting than either treatment alone, while also improves glucose metabolism.

Methods 11-W-old C57BL/6 mice were subjected to hind-limb immobilisation for two weeks, during which ucOC (90ng/g; intraperitoneal) and/or ibandronate (2ug/g; subcutaneous) injections, Insulin tolerance test, and oral glucose tolerance test (OGTT) were performed. After immobilisation, muscles (extensor digitorum longus [EDL], soleus, tibialis anterior, gastrocnemius and quadriceps) were excised, and muscle weight was measured. Glucose uptake in EDL and soleus muscles was assessed. Proteins in anabolic/catabolic pathways were examined in the quadriceps muscle. In addition, older adults-originated primary myotubes treated with ucOC and/or IBN were analysed for the same signalling proteins.

Results Compared with vehicle, individual treatment with ucOC or ibandronate had minimal effect on hind immobilisation-induced muscle wasting ($p>0.05$), while combination treatment increased weight in immobilised soleus (31.7%; $p<0.05$) and quadriceps (20.0%; $p<0.01$) muscles, concomitant with elevated p-Akt (S473)/Akt ratio ($p<0.05$). Although combination treatment had limited effects on muscle glucose uptake in immobilised muscles ($p>0.05$), it enhanced whole-body glucose tolerance assessed via OGTT (16.6%; $p<0.001$). In human myotubes, combination treatment stimulated greater activation of ERK1/2 and mTOR, and led to a lesser expression of MuRF1, than individual treatments (all $p<0.05$).

Conclusions Combination treatment with ucOC and ibandronate exerts protective effects on immobilisation-induced muscle wasting in mice, and regulatory effects on anabolic/catabolic pathways in myotubes of older adults. Furthermore, combination treatment improves whole-body glucose tolerance. These findings suggest a therapeutic potential of combination treatment with ucOC and bisphosphonates for treating muscle wasting induced by immobilisation and ageing.

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PI3K signaling in Dmp1-expressing cells drives periosteal bone formation and bone strength

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Phosphoinositide 3-kinase (PI3K) signaling is an intracellular pathway activated by G protein-coupled and cytokine receptors. Bone formation can be stimulated by elevated PI3K signaling in response to mechanical loading and in some pathologies (e.g. CLOVES syndrome). Our aim was to determine whether PI3K activation specific to the osteoblast lineage could increase bone mass.

We generated a mouse (DPK) where PI3K signaling is activated in osteocytes and endocortical osteoblasts using dentin matrix protein-1 Cre ($Dmp1^{Tg}$) and the *Pik3caH1047R* knock-in allele ($PI3K^{ca}$). We confirmed PI3K activation in bone by Western blot. Micro-computed tomography identified no differences in femoral length between DPK ($DMP^{Tg}.PI3K^{ca/+}$) mice and littermate controls. While there were no detectable changes in trabecular bone, DPK mice had ~50% greater cortical bone mass than age-matched controls at all ages assessed (6-, 12-, 18-, 24-weeks-old). Notably, while bone mass reached a peak and plateaud in control mice, DPK mice continued to accumulate bone in the mature skeleton. Three-point bend tests showed 12-week-old DPK bones had almost double the strength of controls (ultimate force before breaking was 82% greater), indicating that targeted PI3K activation can specifically increase cortical bone strength.

No differences in osteoclast parameters were observed by histomorphometry. Consistent with greater cross-sectional area, periosteal bone formation rate was doubled in DPK mice. This indicates that DMP^{Tg}-targeted PI3K activation specifically increases bone formation at the periosteum. However, by lineage tracing, DMP^{Tg} was not active in periosteal cells, but only in osteocytes and endocortical osteoblasts. Furthermore, RNAseq analysis of isolated cortical bone shafts did not identify any changes in known pathways that stimulate bone formation.

In conclusion, targeting PI3K activation to Dmp1cre+ cells promotes periosteal bone formation indirectly, and without stimulating known bone formation pathways. This suggests a novel mechanism that could inform approaches to stimulate periosteal bone formation and thereby increase cortical bone strength.

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Targeting osteocytes: quantifying their cellular uptake with a high throughput internalisation assay

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An osteocyte-specific targeted delivery system has the potential to improve the efficacy and safety profile of therapeutic candidates for osteoporosis and other skeletal diseases.

Poor cytosolic delivery is a major rate-limiting step in delivery of biological therapies. For effective intracellular drug-delivery, the delivery system should specifically bind to a cell membrane-bound protein and internalise after binding. Although our knowledge of osteocytes and the factors that they release has grown over the last decades, little is known about their ability to internalise materials.

To quantify the binding, internalisation, and recycling of materials delivered to osteocytes, we applied an innovative sensor technology developed by our group known as SHIP (Specific Hybridization Internalization Probe) to osteocyte-like cells. We measured the internalisation kinetics of antibodies against transferrin receptor (TfR), which mediates cellular iron uptake, and E11, which is an osteocyte-specific transmembrane glycoprotein, in undifferentiated (stromal cells) and differentiated (osteocyte-like) Kusa 4b10 cells.

Differentiated Kusa 4b10 cells not only expressed higher E11 levels, but they also had higher uptake (% of total associated antibody - TAA) of the anti-E11 antibody than undifferentiated cells at 0.5, 1, and 2 h after incubation. Differentiated cells also had significantly greater internalisation of anti-TfR antibody (76% of TAA) compared to undifferentiated cells (36% of TAA) as early as 10 minutes after incubation. Only a limited portion of anti-TfR (<20% of TAA) and anti-E11 (<10% of TAA) antibodies were recycled to the surface of differentiated cells during the 40 minutes after initial internalisation, suggesting both antibodies are trafficked into non-recycling endosomes.

Our innovative molecular sensor, SHIP, enables high-throughput and quantitative analysis of the uptake of therapeutics in osteocytes. Our data indicate that osteocytes have greater and more rapid internalisation of anti-E11 antibody than undifferentiated cells without recycling, suggesting that E11 is a promising approach to target drug delivery to osteocytes.

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Unsuppressed STAT3 signaling in osteocytes exaggerates cortical bone response to mechanical load

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Cortical bone develops and changes in response to mechanical load, which is sensed by osteocytes in the skeleton. The bone formation response to load depends on STAT3 intracellular signals, which are upregulated after loading, and are subject to negative feedback from Suppressor of Cytokine Signaling 3 (*Socs3*). Mice with prolonged and elevated STAT3 activation in osteocytes, caused by *Dmp1Cre*-targeted knockout of *Socs3,* have delayed cortical bone maturation. Here we determined whether *Dmp1Cre.Socs3$^{f/f}$*mice have an altered response to physiological mechanical load (treadmill running) and experimental loading (tibial compression).

Daily treadmill running for 5 weeks did not change cortical bone mass in control mice, but further delayed cortical development in *Dmp1Cre.Socs3$^{f/f}$* mice. Cortical bone was thinner than gene-matched sedentary controls and contained less high density bone.

Tibial compression in control (*Socs3$^{f/f}$*) and *Dmp1Cre.Socs3$^{f/f}$*mice increased to a similar extent average cortical bone thickness and periosteal perimeter in the loaded tibia compared to the contralateral limb. However, 360˚ radial analysis revealed that, at the site of greatest compressive strain, the increase in cortical thickness in *Dmp1Cre.Socs3$^{f/f}$*mice was significantly greater than that observed in controls. Calcein labeling confirmed that more new bone was deposited in loaded *Dmp1Cre.Socs3$^{f/f}$*tibiae at both periosteal and endocortical surfaces at this site, than in loaded controls. This included formation of abundant woven bone, similar to that seen when bone is loaded at high strains. This suggests *Dmp1Cre.Socs3$^{f/f}$*mice have a greater sensitivity to mechanical load.

In summary, mice with targeted SOCS3 deletion and immature cortical bone have an exaggerated response to both physiological and experimental loading. We conclude that there is an optimal level of osteocytic response to load required for cortical bone maturation and that load-induced bone formation may be enhanced by limiting the SOCS3 negative feedback loop in osteocytes.

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Bone Quality Assessment in Health and Disease

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An increasing number of patients worldwide suffer from bone fractures that occur after low intensity trauma. Such fractures are usually associated with advanced age and osteoporosis but changes on the bone tissue level happen also with immobilization, Paget's disease of bone, and other metabolic disorders. Revealing the skeletal origins of increased bone fragility is a prerequisite to contribute to improved health monitoring, preventive and therapeutic strategies. In this context, analyzing bone quality indices including osteocyte characteristics in physiological and pathological conditions using functional imaging modalities and microanalyses may inform researchers and clinicians. The characteristics of osteocyte lacunae along the osseous matrix composition distinguishes healthy, aged and diseased bone. Osteocyte lacunar characteristics could be regarded as a vital marker for bone quality assessment and fracture risk.

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Exploring the mechanisms underlying the metabolic improvements following bariatric surgery

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Content coming soon.

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Perturbed BMP signalling and denervation promote muscle wasting in cancer cachexia

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Most patients with advanced solid cancers exhibit features of cachexia, a debilitating syndrome characterized by progressive loss of skeletal muscle mass and strength. Because the underlying mechanisms of this multifactorial syndrome are incompletely defined, effective therapeutics have yet to be developed. Here, we show that diminished bone morphogenetic protein (BMP) signaling is observed early in the onset of skeletal muscle wasting associated with cancer cachexia in mouse models and in patients with cancer. Cancer-mediated factors including Activin A and IL-6 trigger the expression of the BMP inhibitor Noggin in muscle, which blocks the actions of BMPs on muscle fibers and motor nerves, subsequently causing disruption of the neuromuscular junction (NMJ), denervation, and muscle wasting. Increasing BMP signaling in the muscles of tumor-bearing mice by gene delivery or pharmacological means can prevent muscle wasting and preserve measures of NMJ function. The data identify perturbed BMP signaling and denervation of muscle fibers as important pathogenic mechanisms of muscle wasting

associated with tumor growth. Collectively, these findings present interventions that promote BMP-mediated signaling as an attractive strategy to counteract the loss of functional musculature in patients with cancer.

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Effect of Testosterone Treatment on Bone Microarchitecture and Bone Mineral Density in Men: A 2-Year RCT

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Context

Testosterone treatment increases bone mineral density (BMD) in hypogonadal men. Effects on bone microarchitecture, a determinant of fracture risk, are unknown.

Objective

We aimed to determine the effect of testosterone treatment on bone microarchitecture using high resolution–peripheral quantitative computed tomography (HR-pQCT).

Methods

Men ≥ 50 years of age were recruited from 6 Australian centers and were randomized to receive injectable testosterone undecanoate or placebo over 2 years on the background of a community-based lifestyle program. The primary endpoint was cortical volumetric BMD (vBMD) at the distal tibia, measured using HR-pQCT in 177 men (1 center). Secondary endpoints included other HR-pQCT parameters and bone remodeling markers. Areal BMD (aBMD) was measured by dual-energy x-ray absorptiometry (DXA) in 601 men (5 centers). Using a linear mixed model for repeated measures, the mean adjusted differences (95% CI) at 12 and 24 months between groups are reported as treatment effect.

Results

Over 24 months, testosterone treatment, versus placebo, increased tibial cortical vBMD, 9.33 mg hydroxyapatite (HA)/cm^3) (3.96, 14.71), $P < 0.001$ or 3.1% (1.2, 5.0); radial cortical vBMD, 8.96 mg HA/cm^3 (3.30, 14.62), $P = 0.005$ or 2.9% (1.0, 4.9); total tibial vBMD, 4.16 mg HA/cm^3 (2.14, 6.19), $P < 0.001$ or 1.3% (0.6, 1.9); and total radial vBMD, 4.42 mg HA/cm^3 (1.67, 7.16), $P = 0.002$ or 1.8% (0.4, 2.0). Testosterone also significantly increased cortical area and thickness at both sites. Effects on trabecular architecture were minor. Testosterone reduced bone remodeling markers CTX, −48.1 ng/L [−81.1, −15.1], $P < 0.001$ and P1NP, −6.8 µg/L[−10.9, −2.7], $P < 0.001$. Testosterone significantly increased aBMD at the lumbar spine, 0.04 g/cm^2 (0.03, 0.05), $P < 0.001$ and the total hip, 0.01 g/cm^2 (0.01, 0.02), $P < 0.001$.

Conclusion

In men ≥ 50 years of age, testosterone treatment for 2 years increased volumetric bone density, predominantly via effects on cortical bone. Implications for fracture risk reduction require further study.

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New Diagnostic and Therapeutic Options in Vasopressin-Dependent Fluid Disorders

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This talk will discuss new diagnostic and therapeutic options in patients with the polyuria polydipsia syndrome on one side and in patients with the syndrome of inappropriate antidiuresis (SIAD) on the other side.
In polyuria polydipsia syndrome, central diabetes insipidus has to be differentiated from nephrogenic diabetes insipidus and from primary polydipsia. This differentiation is important since treatment differs and a wrong treatment can have serious consequences. For decades, the water deprivation test was the diagnostic gold standard, but its interpretation is often misleading. In this presentation, new osmotic and non-osmotic stimulation tests measuring copeptin, the C-terminal part of the vasopressin precursor will be discussed.
Also, a new medical treatment option in patients with primary polydipsia will be presented.

In patients with SIAD, copeptin as a potential new diagnostic marker in the differential diagnosis of hyponatremia and as a marker to predict neoplastic SIAD will be discussed. Treatment in SIAD is challenging and new treatment options are needed. Data concerning SGLT-2 inhibitors as a novel treatment option in patients with SIAD will be presented.

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Gene Therapy Approaches to Rapidly Manipulate Testis Function

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Recent developments in gene-therapy vector technologies make it possible for rapid design, production, and analysis of complex genetic mouse models, that circumvent traditional breeding, genotyping and maintenance of multiple complex mouse lines. We have characterised multiple technologies to define approaches to selectively deliver genetic constructs to different somatic cell populations of the testis. Using these, we are able to perturb, inhibit, induce, label, track or replace gene function for multiple genes simultaneously in wild-type mice, with analysis possible as little as 48 hours post-treatment.

Our results demonstrate differing applications for alternative vector systems, including for generic and bespoke Lentivirus, adenovirus, adeno-associated virus, and nanoparticles, with or without cell-selective targeting.

Using a combination of these systems, in single treatments we are able to selectively block spermatogenesis (for more than 1 year) or restore spermatogenesis in mutant animals. Or alternatively, selectively inhibit or enhance steroid hormone production by the testis.

Together these approaches provide proof of principle for future methodologies to selectively manipulate testis function in support of, or to suppress, spermatogenesis, and to support lifelong male health through manipulation of androgen production and action.

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Genomics and High Throughput Screening Analysis of Progesterone Receptor Action for Discovering Selective Molecular Targets for Blocking Ovulation

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The human egg, or oocyte, grows to maturity within an ovarian follicle and is then released through the process known as ovulation so it can contact sperm and be fertilised. Ovulation is a unique process during which the follicle is ruptured and cells around the oocyte produce a specialised protein matrix which plays the critical role in carrying the oocyte out of the follicle. A technology that blocks ovulation would produce an ideal contraceptive from efficacy, safety and acceptability considerations because it can be non-hormonal, acute acting and not disturb the other aspects of endocrine physiology that are important for health and wellbeing. Additionally, by preventing fertilisation, there are no ethical concerns around embryo fate. Our team uses genomics tools to fully characterise the hormone actions unique to ovulation. One key mediator of ovulation is Progesterone Receptor (PGR). We use vertical integration of ChIP-seq, ATAC-seq and RNA-seq in human cells and mutant mouse models, to define the specialised transcriptional mechanism of PGR in the ovary and the downstream biomechanical effectors that are essential for ovulation. We are also applying our expertise in the biomechanics of ovulation to develop in vitro surrogate models as screening tools to survey whole genome or large drug libraries as an objective means to identify new critical pathways and lead compounds that specifically block ovulation. The results have uncovered exciting ovary specific mechanisms of steroid hormone signalling and tissue remodelling that present novel targets for ovulation blocking contraceptives.

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Using epigenetics and cell of origin analysis as diagnostics for azoospermia

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Male infertility is a common health issue in men with ever increasing incidence. Unfortunately, available diagnostics are inadequate to drive clinical decision making in the vast majority of cases. New forms of diagnostics are needed to provide more personalized care and to improve outcomes. One area of recent interest is the diagnostic utility of sperm epigenetics. While becoming more common to explore, there are still many unknowns regarding the potential of these marks to drive clinical care. We will discuss the unique epigenetic marks in sperm and how these can potentially be used to provide clinically actionable care for some of the most difficult cases of male infertility, namely, cryptozoospermia and azoospermia.

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Mitochondria, Maternal Inheritance and Implications for Male Reproductive Health

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Mitochondria are maternally inherited; males never pass on their mitochondria to their children**.** This has evolutionary implications because it renders natural selection ineffective at removing mutations in the mitochondrial DNA (mtDNA) when these mutations only effect males. In other words, whether an mtDNA mutation establishes in a population, or goes extinct, depends exclusively on how that mutation affects the chances of females successfully reproducing. If an mtDNA mutation appears that decreases the chances of females reproducing, then the mutation is likely to be removed by selection (i.e., females that carry the mutation are less likely to reproduce than females that do not carry the mutation). Conversely, if an mtDNA mutation arises that increases the chances of females reproducing, selection should favour it, and the mutation should increase in frequency (i.e., females that carry the mutation are more likely to reproduce than females that don't carry it). But, what about the case of a "sex-specific" mtDNA mutation – specifically, a mutation that either has no effects on the reproductive outcomes of females, or even beneficial effects on females, but which severely decreases the chances of males reproducing? Somewhat ironically, selection will classify this mutation as benign or beneficial, since it does not decrease the chances of females reproducing, and that mutation can persist (females will pass on the mutation).

This is, in essence, the evolutionary logic underlying a hypothesis that has come to be known as “Mother’s Curse”, and it predicts that mtDNA sequences will be enriched for mutations that impair male fertility, but which have no negative effects on females.

In this talk, I will first outline this theory, and its key predictions. I will then detail experimental evidence that supports the prediction that these male-sterilising mtDNA mutations may be pervasive in animals. Our experiments come from studies in the fruit fly, *Drosophila melanogaster*, but recent evidence for the Mother’s Curse effect extends to mice, hares and humans. Finally, I will discuss the potential for these foundational evolutionary findings to contribute to real-world applications, focusing on novel approaches to pest control and male contraception.

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Discovery of a key regulator of endometrial receptivity for embryo implantation

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Objective: Embryo implantation is a complex process requiring coordinated interactions between a well-developed embryo and a receptive endometrium. However, the fundamental mechanisms governing endometrial receptivity, particularly at the luminal surface where an embryo first interacts with, are not well understood. This study aimed to identify key factors that control human endometrial epithelial receptivity and to determine their functional importance.

Methods: Primary human endometrial epithelial cells were isolated from women and analyzed by proteomics for adhesion-related membrane proteins. Novel candidates of interest were then investigated for *in vivo* expression pattern and cellular localization in the human endometrium across the menstrual cycle using immunohistochemistry and for hormonal regulation using cell culture. Functional importance was next determined using *in vitro* models of human embryo attachment and invasion, and using endometrial tissues obtained from women undergoing IVF treatment.

Results: Podocalyxin (PODXL) was identified as a novel endometrial epithelial receptivity marker. PODXL was highly expressed on the apical surface of all epithelial and endothelial cells in the non-receptive endometrium, but selectively and specifically down-regulated in the luminal epithelium at receptivity; this down-regulation was confirmed to be mediated by progesterone. In *in vitro* implantation models, endometrial epithelial PODXL inhibited not only the attachment but also the invasion of both human embryo mimics (trophoblast spheroids) and actual human embryos, demonstrating that the above described down-regulation of PODXL is essential for endometrial surface receptivity. Clinically, inadequate luminal epithelial down-regulation of PODXL at the time of embryo transfer was associated with implantation failure in women undergoing IVF treatment.

Conclusions: PODXL is a key and previously unknown regulator of human endometrial epithelial receptivity. PODXL inhibits embryo implantation and its down-regulation in the luminal epithelium opens the window of implantation.

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Defining the heterogeneity and function of macrophages during endometrial tissue repair

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Endometrial repair is essential for women’s reproductive health and ongoing fertility. Macrophages are essential mediators of tissue repair but we lack fundamental knowledge about how they are regulated in the endometrium. Although there is consensus that macrophages are necessary for repair, their nature, origins and the environmental factors that control their behaviour remain poorly understood thereby limiting our understanding of their function in the endometrium in health and disease.

We have used Csf1r-eGFP transgenic reporter mice in which cells of the monocyte/macrophage lineage are labelled with eGFP (MacGreen) to characterise macrophages during endometrial repair. We have shown that CSF1R+ cells are abundant during endometrial repair and found that monocyte and macrophage subpopulations associate with spatially distinct regions of tissue breakdown and repair ([1]). We performed multiparameter flow cytometry on uterine repair tissues using established subset markers for monocytes and mature macrophages and found that theses populations change dynamically during repair with considerable evidence for heterogeneity within the endometrial macrophage compartment. To investigate this further we performed unbiased profiling of CD45+ cells using single cell RNA sequencing which confirmed the presence of multiple monocyte/macrophage subtypes with potentially distinct ontogenies. Further analysis across distinct time points during endometrial breakdown (12hr), repair (24hr) and remodelling (48hr) identified that endometrial repair was associated with extensive monocyte infiltration and that monocytes supersede tissue resident macrophages during active repair. Notably, infiltrating monocytes expressed key genes associated with tissue repair and were enriched for genes associated with wound healing processes. Following resolution of inflammation, mature macrophages were predominant during endometrial remodelling.

This study offers new insight into the regulation of the endometrial macrophage compartment during endometrial repair and provides a platform for understanding how the phenotype and function of these cells could be dysregulated in women’s reproductive health disorders.

1. [1] Cousins, F.L., Kirkwood, P.M., Saunders, P.T.K. & Gibson D.A. Evidence for a dynamic role for mononuclear phagocytes during endometrial repair and remodelling. Sci Rep 6, 36748 (2016). https://doi.org/10.1038/srep36748

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Puma knockout protects the uterus from radiotherapy-mediated damage: implications for fertility preservation for female cancer survivors

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As cancer survivorship rises, addressing the off-target impacts of cancer therapies has become increasingly important. Clinically, previous radiotherapy exposure is associated with reduced pregnancy rates and pregnancy complications. However, specific impacts on the uterus have not been investigated. Here, we aimed to determine if radiotherapy exposure damages the uterus and compromises fertility.

Adolescent (4-week-old) C57BL6/CBA(F1) female mice were untreated or exposed to whole body γ-irradiation (7Gy), then ovariectomised to distinguish uterine from ovarian damage. Within 24 hours, DNA damage (γH2AX) and *Puma*-mediated apoptosis were elevated in uteri (n=4/group), demonstrating immediate, direct uterine damage post-irradiation.

Implantation rates were unchanged between groups after healthy donor embryo transfer (3-days post-transfer; n=11-13/group), although pale, atrophic uteri suggested radiation may impair vascularisation. By 10-days post-transfer, all irradiated mice experienced resorption (control 4.0±1.0 vs. 7Gy 0.3±0.2 viable sites; p<0.01), though ultrasound did not detect changes in uterine artery pulsatility or resistance (n=8-10/group). Wire myography performed on uterine arteries demonstrated endothelial dysfunction in irradiated mice (area under the curve, control 303.7±9.91 vs 7Gy 227.4±10.55, p<0.01; n=9-10/group).

Endometrial receptivity and decidualisation were artificially induced to investigate adaptation to pregnancy. Irradiated mice demonstrated normal receptivity, but lower decidualised uterine:body weight ratio (control 446.0±59.4mg vs. 7Gy 147.3±34.8mg; p<0.01; n=7-8/group). Similarly, primary human endometrial stromal cell decidualisation was lower *in vitro* post-irradiation (prolactin secretion; control 278±42.5pg/mL vs. 7Gy 79.39±42.5pg/mL, n=2/group). Apoptosis-resistant *Puma-null* mice (n=4-5/group) decidualised normally post-irradiation compared to wild-type controls, demonstrating *Puma*-mediated apoptosis post-irradiation contributes to impaired decidualisation, limiting maternal adaptation to pregnancy and leading to pregnancy loss *in vivo*.

These data demonstrate that direct radiotherapy-mediated damage to the uterus persists long-term, impairing uterine adaptations to pregnancy via a multimodal mechanism. This includes uterine artery endothelial dysfunction, impaired decidualisation, and *Puma*-mediated apoptosis. Critically, *Puma* deletion rescues this phenotype, highlighting *Puma* blockade as a potential therapeutic intervention for improving fertility preservation.

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Detrimental actions of obesity-associated Advanced Glycation Endproducts on endometrial epithelial cell proliferation are alleviated by antioxidants and are donor-dependent in human endometrial organoids

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Background:

Global levels of obesity are rising, with 39% of people overweight or obese (WHO). Obese women experience reduced fertility and increased pregnancy risks including preeclampsia. Advanced Glycation Endproducts (AGEs), a proinflammatory modification of proteins exposed to sugars, are elevated in the uterine fluid of obese women versus lean. AGEs compromise both preimplantation embryo development and endometrial cell functions.

Aim: To investigate therapeutics to restore endometrial cell function, and characterise the effect of obesity-associated AGEs on human endometrial epithelial cell organoids (hEEO).

Methods:

Endometrial epithelial cell line (ECC-1) and hEEO were cultured in AGEs equimolar with lean (2 umol/mol lysine) and obese (8 umol/mol lysine) uterine environments. Real time cell analysis (xCelligence) of ECC-1 examined remedial effects of i) 100 uM metformin; ii) antioxidants (10 uM N-acetyl-cysteine, 10 uM N-acetyl-L-carnitine, 5 uM α-lipoic acid); iii) 25 nM RAGE antagonist (FPS-ZM1). hEEO-derived primary epithelial cells exposed to obese AGEs were examined by xCelligence. Multiplex analysis of chemokine & cytokine secretion (inflammatory determinants) in hEEO conditioned medium. CXCL16 profiled by Luminex analysis and glucose by Randox Daytona analyser in uterine fluid of women undergoing IVF.

Results:

Obese AGEs-reduced ECC-1 proliferation (P<0.001) which was successfully restored by antioxidants. hEEO were functionally impacted by obese AGEs, demonstrating a donor-dependent effect on proliferation. AGEs increased secretion of proinflammatory factors associated with poor pregnancy outcomes, including CXCL16 (P=0.04). Uterine fluid CXCL16 correlated positively to BMI (R=0.26; P=0.02) and uterine glucose (AGEs precursor; R=0.74, P<0.0001), but was not significantly different between IVF cycles resulting in pregnancy vs no pregnancy (P=0.46), nor live birth vs miscarriage (P=0.27).

Conclusion:

AGEs promote a uterine inflammatory milieu hostile to implantation. Antioxidants alleviate the effects of AGEs on ECC1 cells, providing a potential therapeutic for obese women. Clinically, reduced uterine AGEs may improve fertility for obese women wishing to conceive.

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A promising future for the novel zinc IUD now confirmed long acting and reversible in rats

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The copper intrauterine device (IUD) is the only long-term, non-hormonal contraceptive available. However, its numerous side effects, including excessive bleeding and cramping, result in a high rate of removal within the first 3 months. We have developed a rat IUD model to examine an alternative contraceptive, a zinc IUD. This model involves inserting IUDs made of copper or zinc into the rat uterine horns then mating with a male of proven fertility and analysing implantation rates. We found that both copper and zinc IUDs provide effective contraception, preventing 100% of pregnancies. This model also demonstrated long-term efficacy of the zinc IUD, preventing pregnancy as rats were mated repeatedly over a 3 month period. Once the zinc IUDs were removed, rats rapidly returned to fertility proving reversibility.

However, the uterus responds differently to each metal. In copper treatments, histological studies identified local endometrial inflammation and metaplasia of the uterine epithelial cells (UECs), which mediate implantation. The zinc IUD did not cause an inflammatory response or metaplasia of the UECs.

Embryos were collected from IUD-treated rats and an embryo survival assay was performed, comparing embryo development from IUD-treated vs. non-treated control horns. The zinc IUD inhibited the development of 94% of embryos with no histological change to the endometrium, suggesting a unique impact of zinc on the early embryo. Whereas 64% of embryos exposed to copper formed blastocysts, indicating this is not the stage at which its contraceptive action occurs.

This study has shown that a zinc IUD provides similar contraceptive efficacy to a copper IUD in this rodent model. However, these IUDs function via different mechanisms and could result in zinc having a reduced side-effect profile. The zinc IUD has proven to be effective long-term and reversible in this rat model, demonstrating its potential as an alternative non-hormonal contraceptive.

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Heterogeneity in the distribution of smooth muscle actin, collagen fibres and immune cells associated with superficial peritoneal endometriotic lesions

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INTRODUCTION: Endometriosis is characterised by lesions composed of “endometrial-like” tissue found in locations outside of the uterus. Fibrosis is a key component of endometriosis, potentially contributing to disease progression, treatment responsiveness and variation in presentation. However, fibrosis is not currently utilised in endometriosis diagnosis, unlike other pathologies including cancer. The aim of this study was to analyse superficial peritoneal endometriotic lesions to determine if distinct lesion subtypes exist based on smooth muscle actin (SMA), collagen and leukocyte patterns, which could aid in improving disease classification.

METHODS: This study employed tissues from 24 patients across the menstrual cycle with histologically confirmed endometriosis. Immunofluorescence was used to demonstrate the CD10-positive stromal area of lesions (n = 271 lesions from 67 endometriotic biopsies), the SMA-positive tissue and the leukocyte population (CD45+ and CD68+) within and adjacent to lesions. Second harmonic generation microscopy was employed to evaluate the morphology of type-1 collagen fibres within and surrounding lesions.

RESULTS: The proportion of tissue occupied by leukocytes, SMA and collagen was low within endometriotic lesions but increased significantly in the adjacent non-lesion tissue. We identified lesions where collagen fibres formed well aligned capsules around endometriotic lesions (defined by the CD10 border), versus lesions where collagen fibre distribution was random. We also observed considerable inter- and intra-patient variability in the morphology of SMA and collagen within and surrounding lesions, which was dependent in-part on the biopsy location and morphology of endometriotic gland profiles.

CONCLUSIONS: These data suggest there is considerable diversity in the presence of immune cells and morphology of SMA and collagen within and surrounding endometriotic lesions, even within individual patients and single biopsies. This heterogeneity presents a challenge to incorporating these cell and tissue types into any new endometriosis classification systems or prognostic approaches.

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A novel mouse model of retrograde menstruation for the study of endometriosis

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Publish consent withheld

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Jagged1 regulates endometrial receptivity in both humans and mice

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The human endometrium undergoes cycle dependent changes and is only receptive to an implanting blastocyst within a narrow window of 2-4 days in the mid-secretory phase. Recent single cell sequencing of human endometrium across the menstrual cycle has identified an abrupt and discontinuous transcriptomic activation in the epithelia in the mid-secretory phase. Such transcriptomic and accordingly functional changes require delicate interplay between a diversity of factors including cytokines and signaling pathways. The Notch signaling pathway members are expressed in human endometrium. We have previously demonstrated that Notch ligand Jagged1 (JAG1) localizes in the endometrial luminal epithelium (LE) and is abnormally reduced in infertile women during receptivity. However, the functional consequences of reduced JAG1 production on endometrial receptivity to implantation of the blastocyst are unknown. This study aimed to determine the role of JAG1 in regulating endometrial receptivity in humans and mice. Knockdown of *JAG1* in both primary human endometrial epithelial cells and Ishikawa cells (endometrial epithelial cell-line) significantly reduced their adhesive capacity to HTR8/SVneo (trophoblast cell-line) spheroids. We confirmed that in human endometrial epithelial cells, JAG1 interacted with Notch Receptor 3 (NOTCH3) and knockdown of *JAG1* significantly reduced the expression of Notch signaling downstream target *HEY1* and classical receptivity markers. Knockdown of *Jag1* in mouse LE significantly impaired blastocyst implantation. Via a customized RT^2 Profiler PCR array, we identified ten genes (related to tight junction, infertility and cell adhesion) that were differentially expressed by *Jag1* knockdown in LE in mice. Further analysis of the tight junction family members in both species revealed that JAG1 altered the expression of tight junction components only in mice. Together, our data demonstrated that JAG1 altered endometrial epithelial cell adhesive capacity and regulated endometrial receptivity in both humans and mice likely via different mechanisms.

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Checkpoint inhibitor associated autoimmune diabetes: an emerging and fulminant form of type 1 diabetes

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Immune checkpoint inhibitors (ICIs) have transformed the landscape of oncological therapy, but at the price of immune related adverse events. Among these, checkpoint inhibitor related autoimmune diabetes (CIADM) entails substantial long-term morbidity due to the complex nature of its glycaemic management.

We sought to characterise the disease phenotype of CIADM. Patients who developed CIADM after ICI use were retrospectively identified across Westmead Hospital, Blacktown Hospital, Melanoma Institute Australia and Royal North Shore Hospital. CIADM was defined as new onset hyperglycaemia (random BGL≧11.1mmol/L or HbA1c≧6.5%) and insulin deficiency (C-peptide <0.4nmol/L). Electronic medical records were reviewed.

25 patients with CIADM were identified, making this the second largest series ever reported internationally. Median age was 66 years (IQR 49-83). 68% of patients had melanoma as the primary malignancy. All patients had either anti-PD1 or anti-PDL1 therapy and 44% had this in combination with anti-CTLA4 therapy. 72% of patients had complete or partial oncological response. Median time from ICI commencement to CIADM onset was 19 weeks. 20% had pre-existing type 2 diabetes requiring diet control and/or oral hypoglycaemic agents only. 60% presented with diabetic ketoacidosis at CIADM onset and required ICU admission. 38% of patients were positive for traditional T1D autoantibodies. 10 patients had HLA typing with 3 patients carrying a T1D-risk haplotype and 2 patients with protective haplotypes for T1D. 28% had elevated lipase at presentation. Infliximab was trialled at diagnosis in one patient but was unsuccessful at reversing insulin dependence. All patients remain on insulin, with 3 patients managed with insulin pumps.

CIADM leads to fulminant T1D with a high incidence of diabetic ketoacidosis. In comparison with T1D, there is a lower incidence of T1D autoantibodies and T1D associated haplotypes.

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Thyroid peroxidase and thyroglobulin antibodies in thyroid immune related adverse events following immune checkpoint inhibitor treatment.

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Objective: The significance of thyroid peroxidase (TPOAb) and thyroglobulin antibody (TgAb) in the pathogenesis of thyroid immune related adverse events (irAEs) and their relationship to cancer survival outcomes are unknown. We studied TPOAb and/or TgAb positivity in thyroid irAEs related to immune checkpoint inhibitor (ICI) treatment.

Design: Retrospective cohort study conducted through Australian academic teaching hospitals.

Patients: Patients with advanced or metastatic melanoma receiving immune checkpoint inhibitor treatment.

Measurements: TPOAb, TgAb and interleukin-6 (IL-6) were measured at baseline and repeated at diagnosis in thyroid irAE patients or 30-60 days after start of ICI-treatment in euthyroid patients.

Results: 122 patients received ICI-treatment. Baseline elevation of TPOAb or TgAb was present in 19 (16%) and 28 (23%) patients, respectively. Patients with overt thyrotoxicosis (n=37) were more likely to have an elevated TPOAb and/or TgAb at baseline and had a higher median TPOAb and TgAb titre compared to patients with subclinical thyrotoxicosis (n=47) or persistent euthyroidism (n=31). Overt thyrotoxicosis was associated with significant increases in TPOAb and TgAb during treatment, which was not observed in patients who remained euthyroid or patients with other thyroid irAE subtypes. Positive TPOAb at baseline was associated with improved PFS in all patients (HR 0.30, 95% CI 0.09-0.99, p=0.04), driven mainly by patients with overt thyrotoxicosis (HR 0.08, 95% CI 0.01-0.79, p=0.03). Baseline IL-6 levels were not associated with thyroid irAE onset but did significantly increase during treatment in patients who developed overt hypothyroidism.

Conclusions: TPOAb positivity at baseline was more prevalent in patients with overt thyrotoxicosis and was associated with improvements in PFS. TPOAb positivity may be a useful biomarker to identify patients at risk of overt thyrotoxicosis irAEs and patients with increased likelihood of response to ICI-treatment.

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Cellular immunotherapy for prostate cancer is a treatment option when AR targeted therapies fail

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Despite significant improvements in detection and treatment, advanced prostate cancer remains incurable when androgen receptor (AR)-targeted therapies fail. New treatment options are needed for these men. The treatment of blood cancers has been revolutionised by new immunotherapy approaches, such as genetically engineered chimeric-antigen receptor (CAR) T cells. Our team has developed 3rd generation CAR T cells that recognize Lewis Y (LeY) glycolipid antigen, which is over-expressed in >50% of solid tumours, including prostate cancer. Here, we evaluated their potential for incurable prostate cancer. In vitro, LeY-CAR T cells induced morphological destruction and propidium iodine (PI) uptake (indicating secondary necrosis); killing was mediated by granule exocytosis mechanism as granzyme/ perforin inhibitors significantly reduced cell death. In contrast, in vivo PDX treatment with LeY -CAR T cells alone did not inhibit tumour growth. However, when combined with carboplatin chemotherapy (but not docetaxel or the anti-PD-1 antibody nivolumab) the combination of treatments reduced tumours to <1% of the starting tumour volume. Residual cancer cells were surrounded by infiltrating T cells, indicating trafficking and persistence of CAR T cells in the combination treatment group vs CAR T cells alone. LeY-CAR T cell therapy is in early phase clinical development for patients with solid tumours, and these studies provide essential preclinical evidence of LeY-CAR T cell efficacy, defining an optimal treatment strategy for clinical trial design.

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Effects of estradiol on cognition in men undergoing androgen deprivation therapy for prostate cancer: a randomised placebo-controlled trial

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Objective: Any role for sex steroids in modulating cognition in men remains uncertain. We used an experimental paradigm that allowed effects of estradiol (E2) on cognition in older men to be investigated, in the absence of testosterone.

Design: Randomised, placebo-controlled trial of E2 for 6 months, hypothesising that men randomised to E2 would have improved verbal learning, verbal memory and spatial problem solving over time, compared to placebo.

Methods: Participants receiving androgen deprivation therapy for prostate cancer were randomised to 0.9 mg of 0.1% E2 gel per day, or matched placebo. Cognition was assessed by a tablet-based cognitive battery at baseline, month 1, month 3, and month 6. Cognitive tests were: International Shopping List (ISL) (verbal learning and memory); Groton Maze Learning (GML) (spatial problem solving); Detection (processing speed, visual attention, psychomotor function); Identification (attention, psychomotor function, information processing speed); One Card Learning (visual memory); and One Back Task (memory and attention). Anxiety and depression were assessed using the Hospital Anxiety and Depression Scale inventory.

Results: 78 participants were randomised. Serum E2 increased in the E2 group over 6 months compared to the placebo group, mean adjusted difference (MAD) 56.5 pg/mL (95% CI 33.6 – 79.4), p<0.001. There was no significant difference in performance over time between the E2 group and the placebo group for the ISL test, MAD 0.7 (95% CI -1.2 – 2.5), p=0.36, or for the GML test, MAD -3.2 (95% CI -12.0 – 5.6), p=0.53. There was no significant difference between groups in performance on any of the other cognitive tests or in depression or anxiety scores.

Conclusion: We found no major effects of E2 on cognition. Although cognitive effects of ADT are debated, this study suggests that any such effects, are unlikely to be prevented by administration of E2.

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Measurements of circulating conjugated and unconjugated vitamin D metabolites by enzyme hydrolysis combined with liquid chromatography mass spectrometry

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Phase II metabolism comprising conjugation reactions by sulfation or glucuronidation are important mechanisms for the inactivation, storage and excretion of endocrine metabolites including vitamin D. Recent studies highlight that 25-hydroxyvitamin D3 (25OHD3) circulates at high levels of phase II metabolites, matching or exceeding the unconjugated form. However, the clinical significance of phase II conjugated 25OHD3 and other vitamin D metabolites remains uncertain as vitamin D status in health is conventionally restricted to measuring unconjugated circulating 25OHD3 plus 25OHD2. This project aimed to determine the proportion of phase II conjugated vitamin D metabolites relative to the unconjugated levels in circulation.

An optimized enzyme hydrolysis method by recombinant arylsulfatase and beta-glucuronidase combined with ultrapressure liquid chromatography mass spectrometry (LC-MS/MS) was validated to estimate the proportions of vitamin D sulfate and glucuronide conjugates. Total conjugated and unconjugated forms of four vitamin D metabolites (25OHD3, 25OHD2, 3-epi-25OHD3, 24,25$(OH)_2$D3) in 170 human samples were categorised by vitamin D supplementation status.

Sulfate conjugates comprised between 18-53% and glucuronide conjugates between 2.7-11%. The proportion of total circulating conjugated forms varied between vitamin D metabolites; 25OHD3 48±9%, 25OHD2 29±10%, 3-epi-25OHD3 30±8%, and 24,25$(OH)_2$D3 62±10%. Although conjugated metabolites correlated with unconjugated forms (r=0.85 to 0.97) the relationship differed between metabolites. This study reveals that vitamin D and its metabolites circulates largely as phase II, mainly sulfate, conjugates with metabolites varying in their proportion of conjugated forms. This optimised method highlights the importance of combining both conjugated and unconjugated measurements for a comprehensive assessment of vitamin D status in health.

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Ablation of normal vitamin D signalling impairs skeletal muscle regeneration in mice

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Satellite cells are muscle stem cells that differentiate into myoblasts and then myocytes. Normally quiescent in adult muscle, satellite cells can be induced to proliferate for regeneration of muscle in response to injury. Our previous work on mice with myocyte-specific deletion of the vitamin D receptor (mVDR) found that these mice had abnormal muscle function and physiology. In this study, we used mice with deletion of VDR in satellite cells (sVDR) to investigate whether vitamin D signalling is important for muscle regeneration and repair after acute injury.

Floxed VDR mice were bred with mice expressing Cre-recombinase driven by the Pax7 promoter to generate sVDR mice and their floxed control (FC) littermates. Pax7 is expressed in satellite cells after birth. Notexin (0.1µg/mL), a myotoxin, was injected into the left tibialis anterior (TA) muscle of 6-months old female sVDR mice. The contralateral TA was injected with 0.9% saline and served as control in each mouse. Mice were euthanised after 10 days.

At 10 days, saline-injected TA in sVDRs were of similar size to FC. However, notexin treated muscles from sVDR mice were 22% smaller (P=0.0004). Notably, notexin-treated TA in FCs were 15% heavier than their matching saline-control TA (P=0.02). This effect was not observed in the sVDR mice. Assessing the size distribution of myofibres in TA, notexin treated TA in control mice had a higher population of myofibres with larger cross-sectional area (FC=5.57% vs KO=0.99%, P=0.03), and an increased proportion of fibres containing centralised nuclei (FC=59.9% vs KO=47.3%, P=0.02).

Overall, these results demonstrate an important role for vitamin D in muscle regeneration. Centralised nuclei are indicative of muscle fibres undergoing repair. Together, the larger sized myofibres and higher number of centralised myonuclei in FC mice suggest further progression of repair in muscles with normal vitamin D signalling.

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Aberrant Igf2-H19 Expression in the Placental Endocrine Zone Increases the Susceptibility of the Mother to Poor Metabolic Health

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Background: During pregnancy, the mother must adapt metabolically to support offspring growth. The placenta secretes hormones with metabolic effects, although the precise role of its endocrine function in determining maternal health is largely unknown. Previous work has shown that the imprinted *Igf2-H19* locus is involved in controlling placental endocrine function and conceptus development in mice. This study used conditional mis-expression of the *Igf2-H19* locus, through deletion of the imprinting control region ICR1, to induce placental endocrine malfunction and study its consequences for maternal metabolism.

Methods: Transgenic mice were crossed to produce entire litters with reduced levels of the *H19* gene and activation of the normally silent maternal *Igf2* gene in the placental endocrine zone (*H19DMRflox/TpbpaCre*; Jz-ICR1D). On day 16 of gestation, maternal blood was collected for metabolite analysis, maternal liver for RNAseq analysis, and placentas collected for endocrine cell culture experiments followed by LC-MS on the conditioned media. Data were compared to dams with unaltered placental *Igf2-H19* locus expression.

Results: Jz-ICR1D dams had heavier kidney, heart and liver weights compared to pregnant controls. They also displayed higher circulating levels of glucose, insulin, LDL-cholesterol, leptin, progesterone, estradiol and corticosterone. RNAseq analysis showed that genes involved in translation, mitochondrial homeostasis and response to oxidative stress, among others were affected in the maternal liver by Jz-ICR1D. Circulating AST, which indicates liver damage, was lower in dams with Jz-ICR1D. A total of 744 proteins were identified in the media from placental endocrine cell cultures, of which 92 were exclusively detected in the Jz-ICR1D group with 30 previously identified to be secreted. The placental endocrine zone was increased by Jz-ICR1D, but maternal plasma IGF2 and fetal growth were unchanged.

Conclusion: Genetically-induced expansion of the placental endocrine zone alters maternal body composition and whole-body metabolic function in pregnancy.

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Mechanisms of Xenobiotic-Induced Ovarian Toxicity

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The female gonad is detrimentally affected by a variety of environmental exposures including polycyclic aromatic hydrocarbons, anti-cancer therapies, pesticides, plasticizers and persistent organic pollutants. Mechanisms of ovotoxicity include hyperactivation of primordial follicle growth, somatic and germ cell DNA damage, impaired folliculogenesis and steroidogenesis. The ovary can respond to ovotoxicants by chemical biotransformation and DNA damage. Despite this, ovotoxicant exposures can result in endocrine disruption and early onset of menopause. We have discovered a physiological status x exposure paradigm of ovotoxicity, such that altered systemic metabolism can influence ovotoxicity. Specifically, the ovary of an obese female has blunted chemical biotransformation and DNA repair responses to ovotoxicant exposures. Perfluorooctanoic acid (PFOA) is a persistent environmental pollutant with a half-life of years in humans. To explore the hypothesis that altered systemic physiology during obesity would affect the ovarian response to PFOA exposure, female wild type (KK.Cg-a/a; lean) or KK.Cg-Ay/J mice (obese) received saline (CT) or PFOA (2.5 mg/Kg) per os for 15 days beginning at 7 weeks of age with water and food ad libitum. There were no treatment effects on food intake, final body weight, steroid hormone level, length spent at different stages of the estrous cycle, uterus, heart, kidney, or spleen weight ($P > 0.05$). Liver weight was increased ($P < 0.05$) by PFOA exposure in both lean and obese mice. Ovary weight was decreased in the lean but not obese mice exposed to PFOA ($P < 0.05$). Relative to vehicle control, exposure to PFOA altered 22 and 28 proteins in lean and obese mice, respectively, as quantified by LC-MS/MS. Cellular pathways targeted by PFOA included cancer, estrogen signaling, PI3K-AKT signaling, progesterone-mediated oocyte maturation, metabolic, xenobiotic metabolism, DNA damage and reproduction. Taken together, ovotoxicants negatively affect fertility through several modes of action and alterations to systemic metabolism contribute to ovotoxicity.

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Differential Follicle Stimulating Hormone Glycosylation Modulates Pre-Antral Follicle Growth and Survival Rates

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Ovarian ageing is a naturally occurring physiological process, marked by dynamic changes in ovarian function and hormone secretion. A key endocrine regulator of ovarian function is the heterodimeric glycoprotein hormone, follicle stimulating hormone (FSH). FSH is secreted as two glycosylation variants: partially glycosylated FSH (FSH21) and fully glycosylated FSH (FSH24). These variants have different in-vitro activities, with FSH21 more bioactive than FSH24. Interestingly, analysis of human pituitary extracts has shown that the ratio of FSH21:FSH24 changes with age, with FSH21 predominant in women of reproductive prime, and FSH24 predominant in menopausal women. However, how differential FSH glycosylation modulates ovarian functions remains unknown. This study therefore aimed to determine the effects of FSH21 and FSH24 on follicle growth and survival. To

do this, mouse ovarian follicles were isolated from 3-5wk-old-C57/BL6 mice and treated +/- 10ng/ml, FSH21 (n=85), FSH24 (n=80), a ratio of FSH21:FSH24 at 80:20 (to mimic reproductive prime; n=77), FSH21:FSH24 at 50:50 (n=53), or FSH21:FSH24 at 20:80 (to mimic late peri-menopause; n=78). Follicles were cultured for up to 96hrs and imaged daily to evaluate follicle morphology, and were snap frozen at 24-hour time intervals for qPCR analysis. In the presence of FSH21 dominant conditions, follicle growth was markedly increased at all time points, in comparison to control and FSH24 alone and 20:80 FSH21:FSH24 conditions. Treatment of follicles with FSH24 or 20:80 FSH21:FSH24 resulted in increased basement membrane rupture and oocyte extrusion, with survival rates significantly decreased. qPCR analysis revealed markers of apoptosis were increased in follicles treated with FSH24 alone and 20:80 FSH21:FSH24, while FSH-responsive genes including hormone receptors and steroidogenic enzymes were increased in FSH21 or 80:20 FSH21:FSH24 conditions. These data suggest that the nature of FSH glycosylation modulates the follicular microenvironment to control follicle growth and survival.

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Human *INHBB* gene variant (c.1079T>C:p.Met360Thr) alters testis germ cell content, but does not impact fertility in a mouse model

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Testicular derived inhibin B (α/β_B dimers) acts in an endocrine manner to suppress pituitary production of follicle stimulating hormone (FSH), by blocking the actions of activins ($\beta_{A/B}/\beta_{A/B}$ dimers). This hypothalamic-pituitary-gonadal (HPG) axis is integral to reproductive function, and consequently, imbalances in inhibin/activin can impact gonadal function and fertility. In a recent study, we identified a homozygous genetic variant (c.1079T>C:p.Met360Thr) arising from uniparental disomy of chromosome 2 in the *INHBB* gene (encoding the β_B-subunit of inhibin B and activin B) in a man suffering from infertility (azoospermia). In this study, we aimed to test the causality of the p.Met360Thr variant in *INHBB* and male infertility. Here, we used CRISPR/Cas-9 technology to generate *Inhbb*$^{M364T/M364T}$mice, where mouse INHBB amino acid p.Met364 corresponds with human p.Met360. Surprisingly, it was found that the testes of male *Inhbb*$^{M364T/M364T}$mutant mice were significantly larger compared with those of aged-matched wildtype littermates at 12 and 24 weeks of age. This was attributed to a significant increase in Sertoli cell and round spermatid number and, consequently, seminiferous tubule area, in *Inhbb*$^{M364T/M364T}$males compared to wildtype males. Despite this testis phenotype, male *Inhbb*$^{M364T/M364T}$mutant mice retained normal fertility, daily sperm production levels, and sperm motility. Serum hormone analyses however, indicated that the *Inhbb*^{M364T}variant resulted in reduced circulating levels of activin B, but did not have significant consequences for FSH production. We also examined the effect of this p.Met360Thr, and an additional *INHBB* variant (c.314C>T: p.Thr105Met) found in another infertile man, on inhibin B and activin B *in vitro* biosynthesis. Intriguingly, it was found that both *INHBB* variants resulted in a significant disruption to activin B *in vitro* biosynthesis. Together, this analysis supports that *INHBB* variants that limit activin B production have consequences for testis composition in males.

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The differential distribution of mononuclear phagocyte populations in the immature and adult murine epididymis is consistent with differences in localised immune responses

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The epididymis provides protection to sperm entering the epididymal duct against autoimmune damage, but in contrast must also protect itself against ascending pathogens This requires tolerance to sperm autoantigens in the caput involving anti-inflammatory intra-epithelial dendritic cells and macrophages (mononuclear phagocytes; MPs), however the cauda is much more susceptible to inflammatory damage. To better understand the basis for these regional differences, epididymal MP subsets were examined in immature (25 day-old, prior to mature sperm appearance) and sexually mature (56 day-old) mice possessing a fluorescent transgene inserted into gene loci of the MP-specific proteins, CX3CR1 (CX3CR1-GFP) or CD11c (CD11c-YFP). Tissues were fixed (4% paraformaldehyde), frozen-embedded in OCT and sectioned (10 µm). The pan-macrophage antigen, F4/80, was co-localised by indirect immunofluorescence. Sections were imaged by Olympus VSI 120 slide scanner and cells counted using Fiji software. The majority of intra-epithelial MPs were positive for F4/80, and CX3CR1 and/or CD11c at both days 25 and 56. These cells displayed extensive intra-epithelial cytoplasmic projections and were most abundant in the caput. Intra-epithelial projections were considerably less extensive in the adult corpus and cauda, and throughout the immature epididymis. F4/80+CX3CR1-, F4/80+CX3CR1+, F4/80+CD11c- and F4/80-CD11c+ macrophages with classical polygonal morphology were observed within all interstitial regions but were most prominent in the corpus and cauda at both ages. Interstitial F4/80+CX3CR1+ MPs were more numerous (2-3-fold) than F4/80+CX3CR1- MPs within the caput region, but approximately equal numbers of F4/80+CX3CR1- and F4/80+CX3CR1+ MPs were present within the cauda interstitium. These major differences in number and

distribution of MPs between caput and cauda suggest that the intra-epithelial F4/80+CX3CR1+ MPs predominant within the caput may contribute to the anti-inflammatory environment in that region. Conversely, the single positive F4/80+CX3CR1-, which are most abundant within the interstitium of the corpus and cauda, areas more susceptible to inflammation, may contribute to immune surveillance.

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Understanding ATRX role in a specific PML Nuclear Body in testis of a mouse model of ATR-X syndrome

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The alpha thalassemia, mental retardation, X-linked (ATR-X) syndrome is a difference of sex development affecting XY individuals caused by mutations in the chromatin remodelling gene *ATRX*. Patients display genital abnormalities varying from hypospadias to ambiguous genitalia to male-to-female sex reversal. Gonadal histology reveals small testes containing only a few seminiferous tubules. To investigate the underlying mechanisms of this phenotype, our lab generated mice with *Atrx* specifically inactivated in Sertoli cells (Sc*Atrx*KO), a cell lineage crucial for seminiferous tubule formation. Sc*Atrx*KO mice showed small testes with fewer tubules because of G2/M cell cycle arrest and apoptosis of Sertoli cells during fetal life.

We wanted to understand why ATRX-deficient Sertoli cells of Sc*Atrx*KO undergo apoptosis. We identified a single giant speckle in the nuclei of these cells which expressed GATA4. Unique to Sertoli cell nuclei, GATA4 foci were identified as PML nuclear bodies (PML NBs) in the wildtype and Sc*Atrx*KO gonads. GATA4-PML NBs are composed of a Y chromosome repetitive DNA, ATRX and DAXX which establish a heterochromatic state of the short arm of the Y chromosome. Although formation of PML bodies is independent of ATRX, the loss of ATRX in Sertoli cells affects the size, protein composition and chromatin regulation of GATA4-PML NBs. The loss of ATRX results in the absence of DAXX, HP1α, and PH3 from the GATA4-PML NBs, leading to the loss of the heterochromatic state, chromatin decondensation and vulnerability to DNA damage. This would explain the reported cell cycle arrest in the late G2- mitosis phase, and consequent apoptosis of Sertoli cells.[1] In turn loss of Sertoli cells affects testis cord integrity, resulting in the small testes phenotype seen in the murine model and reported in boys with ATR-X syndrome.

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Transcriptomic analysis of the seminal vesicle response to the reproductive toxicant acrylamide

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The seminal vesicles synthesise bioactive factors that support gamete function, modulate the female reproductive tract to promote implantation, and influence developmental programming of offspring phenotype. Despite the significance of the seminal vesicles in reproduction, their biology remains poorly defined. Here, we analysed the mouse seminal vesicle transcriptome under normal physiological conditions and in response to acute exposure to the reproductive toxicant acrylamide. Mice were administered acrylamide (25 mg/kg bw/day) or vehicle control daily for five consecutive days prior to collecting seminal vesicle tissue 72 h following the final injection. A total of 15,304 genes were identified in the seminal vesicles with those encoding secreted proteins amongst the most abundant. In addition to reproductive hormone pathways, functional annotation of the seminal vesicle transcriptome identified cell proliferation, protein synthesis, and cellular death and survival pathways as prominent biological processes. Administration of acrylamide elicited 70 differentially regulated (fold-change ≥ 1.5 or ≤ 0.67, adjusted *p* value ≤ 0.1) genes, several of which were orthogonally validated using quantitative PCR. Pathways that initiate gene and protein synthesis to promote cellular survival were prominent amongst the dysregulated pathways. Inflammation was a key transcriptomic response to acrylamide, with the cytokine, *Colony stimulating factor 2* (*Csf2*) identified as a top-ranked upstream driver. *Early growth response* (*Egr1*), *C-C motif chemokine ligand 8* (*Ccl8*), and *Collagen, type V, alpha 1* (*Col5a1*), were also identified amongst the dysregulated genes. Additionally, acrylamide treatment led to subtle changes in the expression of genes that encode proteins secreted by the seminal vesicle, including the complement regulator, *Complement factor b* (*Cfb*). Together these findings support the interpretation that toxicant exposure influences male accessory gland physiology and highlights the need to consider the response of all male reproductive tract tissues when interpreting the impact of environmental stressors on male reproductive function.

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The male reproductive system is aiding our understanding of how wireless communications networks may be impacting our health

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Wireless communication devices and their networks are an essential part of our civilisation. This now ubiquitous technology has produced a new and persistent presence of radiofrequency and millimetre wave electromagnetic energy in our environment. With ever-accelerating public uptake and technological developments driving wider coverage and faster, more reliable data transmission, community concern about the safety of these devices and networks continues to rise. The research field has identified that the non-ionising energies utilised by wireless communications are not necessarily inert to living organisms, however the biological interactions and potential health implications are still unclear and under robust debate. Indeed, progress toward ruling out significant health risks remains slow and therefore, the public demand for a definitive health risk assessment of these technologies continues to go unmet. We have confirmed that spermatozoa are negatively affected by environmentally relevant electromagnetic fields, and that their distinctive cell biology, provides a unique sensitivity from which we can dissect out the molecular targets and the downstream perturbations of communication fields on biology. Building on our previous work in which we have implicated an oxidative stress cascade initiated by the sperm mitochondria, we have now identified that protein cysteine hyper-oxidation, may be a key mediator precipitating damage and loss of sperm function, including motility losses, that we commonly observe after irradiations. These functional losses including elevated DNA damage, are eliminated by buffering the cell against cysteine hyper-oxidation through supplementation of thiol-based antioxidants such as penicillamine. These insights into sperm thiol dysregulation, offers a clear target of focus for advancing a coveted mechanism of action, and on the backdrop of the recent global decline in human semen parameters that may stem from new prominent factors in our environment, provides a potential intervention strategy that could contribute to combatting waning semen profiles and male fertility.

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Testis development in marsupials is delayed until after birth and may be the driving force behind the unique marsupial mode of reproduction.

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Marsupials typically have a very short gestation period and give birth to highly-altricial young. These young further mature during a prolonged lactation period, usually within a pouch. Why marsupials have not evolved a prolonged pregnancy has remained a mystery.

We examined testis development in the fat-tailed dunnart (Sminthopis crassicaudata). This species of dunnart has one of the shortest gestations of any mammal, just 13.5 days. They give birth to around 10 young which weigh just 12mg each. We found that testis cord formation in this species occurred right after birth, similar to the timing of testis formation in the tammar wallaby. However, the tammar – although still precocial at birth for a mammal – has a 26.5 day gestation and young weigh around 600mg . This is almost 50 times the size of a dunnart neonate, and tammar young are far more advanced in terms of their development and organogenesis. In fact, testis formation seems to be developmentally tied to the timing of birth in all marsupials examined, regardless of their size or developmental stage. Together with our previous work, which has shown that estrogen can drive female development in XY gonads in marsupials, we propose that circulating maternal estrogen may prevent testis formation occurring during gestation. Therefore, the unique marsupial mode of reproduction may have evolved to enable the offspring to complete sex determination outside of the maternal environment.

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Using Statistical Genetics Methods in Large Cohorts to Identify Modifiable Risk Factors for Osteoporosis.

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Genomics is undergoing a dramatic evolution due to rapid advances in statistical genetics methods, the development of new high-throughput sequencing technologies, and the establishment of large population-based biobank studies. New statistical methodologies are allowing us to quantify the degree to which genome-wide arrays tag heritable variation of skeletal disease traits, and identify which regions of the genome (i.e., loci) are most likely to be implicated. Genetic loci discovered using genome-wide association studies are finding a novel use in a technique called Mendelian Randomization, in which genetic variants are used to inform causality in observational epidemiological studies. Moreover, information from genome-wide association studies can also be integrated with single cell transcriptomics analyses to reveal new skeletal disease genes, candidate drug targets, and generate hypotheses regarding the cellular context through which they may function. In this talk I describe many of these new advances in genomics in reference to my team's work on the genetics of bone mineral density and osteoporosis and illustrate how some of these techniques can be used profitably by epidemiologists and molecular biologists.

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Drug Trials In Skeletal Dysplasias

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The management of skeletal dysplasia (inherited disorders of bone and cartilage) has been predominantly, like many other genetic conditions, symptomatic and reactive. This status quo is now being challenged by the promise of precision therapies, underpinned by advances in the understanding of disease pathogenesis, that can potentially alter the natural history of these disorders, and offer patients and families new options for better health.

To assess the safety and clinical efficacy of these new disruptive products, we have been undertaking clinical trials in children with various forms of skeletal dysplasia. This talk will summarise the current state of play with these phase II and III drug trials, and will focus on potential new therapies for children with achondroplasia

(including c-natriuretic peptide, tyrosine kinase inhibitors, soluble FGFR3), and Schmid metaphyseal dysplasia (repurposing carbamazepine as exemplars of this brave new paradigm.

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Paternal Environmental Exposures and Their Influence on Development via the Sperm Epigenome

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Despite the father transmitting half the heritable information to the embryo the focus on preconception health has largely been on the mother. New studies highlight the role of the father in disease transmission via non-genetic inheritance, through epigenetic mechanisms. Epigenetic mechanisms include, DNA methylation, post-translational modifications of histones and noncoding RNAs. Paternal effects have been linked to developmental abnormalities and complex diseases such as cancer, diabetes and obesity. Studies in humans and animals have linked epigenetic inheritance to the transmission of environmentally induced phenotypic traits from the father to the developing embryo and these have been associated with altered gene expression and developmental abnormalities in first and second offspring generations. Our most recent studies of sperm chromatin indicate that environmental challenges can alter the sperm epigenome in a cumulative manner to negatively impact embryo development. In translational studies we have determined that a man's BMI, can alter the sperm epigenome at regions that are implicated in fertility and embryo development. Moreover, in a South African population of men exposure to DDT is associated with alterations to the sperm chromatin, occurs at genomic regions that persist in the pre-implantation embryos at genes that are implicated development including neurodevelopment. These findings indicate that paternal exposures may influence fertility and child health. Additionally, they underscore the need to amplify in depth pre-conception advice for youth and men.

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Residential proximity to bushfire events in Australia impaired subsequent human oocyte *in vitro* fertilization rates

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Climate change is currently the greatest threat facing human health, worldwide. The global incidence and severity of bushfires is increasing, particularly in Australia, causing growing risks of smoke inhalation. While there are overlaps in the chemical makeup between bushfire, industrial or cigarette smoke, notably, bushfires can release unique compounds. Defining the specific impacts of bushfire smoke exposure on reproductive function has important consequences for population size and health; essential inputs to model the potential future health burdens associated with climate change.

The aims of this retrospective cohort study were to examine the association between human oocyte retrieval rates with residential proximity to largescale Victorian fire events during the Australian 'Black Summer' bushfires of 2019-2020, compared with women from distal areas to fire events. All data was stratified based on oocyte retrieval either before, or after the bushfires from December 2019 – January 2020.

Residing from postcodes proximal to bushfire zones was associated with a significant 18.8-23.4% reduction in the proportion of oocytes fertilized from all mature oocytes retrieved (mean 34.8%; 95% confidence interval [CI], 22.5, 47.0) versus all other groups. Rates of blastocysts from mature oocytes retrieved was significantly reduced by 8.8% in women from exposure areas post-fires (mean 13.3% [CI] 7.3%, 19.2%), compared to non-exposure areas post-fires (mean 22.1% [CI] 21.0%, 23.2%). Notably, while there were two clinical pregnancies of the cycles in women from exposure areas post-fires, this was the only group to record no live births. This association between smoke exposure and oocyte retrieval may be an important factor to consider in future research.

Exposure to bushfire smoke was associated with reduced retrieval of quality oocytes that successfully underwent fertilization. These results raise concern that increasing incidence and severity of bushfire events worldwide may result in accelerated reproductive aging among women.

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Clinical experience of spermatogenesis induction for hypogonadotrophic hypogonadism in a tertiary hospital andrology service.

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Infertility affects 8-12% of couples with male factors contributing to 50% and solely responsible in 20-30% of cases. Male infertility due to hypogonadotrophic hypogonadism (HH) (congenital or acquired) is amenable to treatment.

Since 2010, we have treated 21 men with a median age of 31 years. Causes of HH have included panhypopituitarism (n= 10) [empty sella (2), adenoma (n=3), craniopharyngioma (n=1), pituitary hypoplasia (n=3), CHARGE syndrome (n=1), cranial radiotherapy (n=1)], Kallmann syndrome (n=1), thalassaemia major (n=1) and idiopathic (n=8).

Urinary (Pregnyl®) or recombinant (Ovidrel®) human chorionic gonadotrophin (hCG) as an LH substitute was used based on established protocols. Commencing doses were 1500 IU or 62.5mcg s/cut twice weekly respectively and titrated to serum testosterone. Recombinant FSH was added after 6 months for persisting azoospermia. Wherever possible, our protocol for men previously treated with long-acting injectable testosterone was to move to transdermal testosterone for a minimum of 6 months before gonadotrophin therapy was initiated.

There was variability in time to first appearance of sperm (median 16 (range 4 – 29) months). In androgen treatment-naive men with acquired HH in adulthood, sperm was detected within 8 months for most (n=7) and for 3 men, hCG monotherapy was sufficient. For men with congenital causes of HH, time to first sperm detection was longer (median 19 (12 – 22) months). In our limited cohort, for intramuscular testosterone treated men time to spermatogenesis was a median of 17 (12-22) months and 21 (19 – 22) months for the men treated with transdermal testosterone. To date, 7/15 men actively seeking fertility have had children, with 6 requiring assisted reproduction.

Gonadotrophin therapy is an effective fertility treatment for men with HH. Clinicians should consider longer spermatogenesis times in androgen-treated men and those with congenital HH, together with female factors and high rates of assisted reproduction when counselling.

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Severity of obesity impacts adverse maternal and neonatal outcomes independent of pre-existing or pregnancy-related diabetes

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Background: Obesity in pregnancy is known to increase the maternal, neonatal and childhood risks of adverse outcomes. We aimed to determine the influence of obesity class on maternal and perinatal outcomes and to explore the contribution of other maternal factors including age, country of origin, parity, presence of pre-existing and current diabetes disorders and hypertension on outcomes such as large-for gestational age (LGA), small for gestational age (SGA) and neonatal hypoglycaemia.

Methods: We retrospectively analysed data from all singleton births from obese mothers from 2013 – 2017 in Northern Sydney Local Health District in Sydney, NSW, Australia. Maternal obesity was categorized into obesity class I (BMI 30-34.9), class II (BMI 35-39.9) and class III and above (BMI 40+). The primary outcomes were LGA and SGA neonatal size. The secondary outcomes were neonatal hypoglycemia, birth defect and timing of birth. Univariate and multivariate logistic analysis model were used to explore the impact of maternal cofounders on neonatal outcomes.

Results: Of 2466 births to obese women, neonatal LGA was more likely in women with obesity class III and above vs class I (OR=1.47, 95% CI 1.03-2.08, p=0.04), multiparous vs nulliparous (OR=1.52 – 1.78, 95% CI 1.19-2.62, p=0.01), and age over 40 vs age under 25 years old (OR=1.86, 95% CI 2.16-2.99, p=0.01). In the obesity class III and above group, women with pre-existing gestational diabetes had more than one-and-half times higher risk of LGA than women without pre-existing GDM (OR=1.66, 95% CI 1.09-2.54, p=0.03). Birth defects were increased more than two-fold for women with obesity class III and above compared with class I (OR=2.17, 95% CI 1.01-4.66, p=0.045).

Conclusion: Increasing maternal obesity class increases risk of adverse perinatal outcomes including delivering an LGA neonate and birth defects, independent of maternal comorbidities such as diabetes and age.

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Altered bone mineral content and body composition in children and adolescents with confirmed prenatal alcohol exposure

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BACKGROUND: Prenatal exposures can contribute to long term impacts on health and disease. We sought to characterise bone and body composition in children and adolescents diagnosed with, or at risk of, fetal alcohol spectrum disorder (FASD) compared to typically developing children.

METHODS: Bone and body composition were determined through use of dual X-ray absorptiometry, and height and weight collected through use of a stadiometer and digital scale respectively. FASD diagnosis was confirmed by a clinical assessment team, including a psychologist, paediatrician, and occupational therapist.

RESULTS: Children with FASD or at risk of FASD (aged 4-10, n=13) tended to be shorter than age matched controls (n=34) ($p=0.05$), although there were no statistically significant differences in other general clinical or densitometric measures. By adolescence (aged ≥11) those with FASD (n=10) remained shorter ($p<0.05$) and recorded lower areal bone mineral density ($p=0.06$) than their typically developing peers (n=26). Multiple regression analysis accounting for age and sex demonstrated a diagnosis of FASD or 'at risk of FASD' was a significant predictor of reduced bone area ($p<0.05$) and a trending predictor of reduced lean tissue mass ($p=0.097$) in the 4-10 age group. From 11 years of age, reductions in bone mineral content ($p<0.05$), and lean tissue mass ($p<0.05$), as well as a greater percentage fat mass ($p<0.05$) were observed in the FASD group.

DISCUSSION: Adolescents who were diagnosed with FASD had greater odds of impairments to bone and body composition. The results suggest that alcohol-induced changes to the regulation of lean tissue mass and bone and fat deposition worsen in the second decade of life, coinciding with body composition changes that occur around puberty. The exacerbation of metabolic outcomes at adolescence highlights the importance of early screening and diagnosis of FASD to allow for interventions in order to optimise bone and body composition.

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Functional genomics for the discovery and characterisation of genes responsible for premature ovarian insufficiency in Perrault syndrome

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Premature ovarian insufficiency (POI), affecting as many as 1 in 100 women, is characterised by menstrual disturbance and elevated follicle stimulating hormone before the age of 40. The condition is highly heterogeneous with over 50 causative genes but these genes only account for ~25% of patients. POI can be associated with significant co-morbidity depending on the underlying genetic pathology. For example, POI is associated with sensorineural hearing loss in individuals with Perrault syndrome. We have used functional genomics to investigate the genetic cause of POI in a large cohort of patients, including ten cases of Perrault syndrome from seven different families. We identified novel causative variants in known Perrault syndrome genes as well as causative variants in *TFAM, GGPS1, PEX6* and *MRPL50*, not previously associated with POI or Perrault syndrome. The role of these genes in ovarian pathology has been consolidated by the identification of additional affected families and/or functional assays in patient fibroblasts or animal models. Most identified genes responsible for Perrault syndrome have a role in mitochondrial translation or peroxisomal biogenesis/function. This highlights the need to consider ovarian function in individuals with atypical/mild mitochondrial and/or peroxisomal disorders. In one family, the affected patient presented only with POI, but genomics revealed causative variants in a Perrault syndrome gene. This demonstrates the utility of using genomics for the investigation and management of POI because co-morbidity can be predicted and the appropriate healthcare team assembled. In this case an audiologist was recruited for optimal patient management. Our genomic study highlights the diverse molecular landscape of POI and Perrault syndrome, and demonstrates the pivotal role mitochondria and peroxisomes play in ovarian function.

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Is it time to rethink the criteria for diagnosing PCOS?

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Aim: Adding polycystic ovary morphology (PCOM) as a criterion for PCOS (Rotterdam criteria) to the NIH criteria of menstrual dysfunction and hyperandrogenism doubles the number of women diagnosed with PCOS, the increase due to the combination of hyperandrogenism and PCOM. We have explored whether hyperandrogenism and PCOM function as independent diagnostic criteria.

Participants: 794 non-healthcare-seeking, euthyroid, normo-prolactinemic, women, aged 18-39 years, not recently pregnant, breast feeding or using systemic hormones.

Measurements: Modified Ferriman-Gallwey scores (mFG), sex steroids measured by LCMS/MS and anti-mullerian hormone (AMH) measured by Beckman Access 2-assay. PCOS was determined using the NIH and Rotterdam criteria for a subset who had a transvaginal ultrasound (TVU).

Results: Serum AMH was independently, positively associated with testosterone and androstenedione, adjusted for age, BMI and smoking (quantile regression β-coefficients 20.90, 95%CI 13.79-28.03; p<0.001and 5.90, 95%CI 3.76-8.03; p<0.001, respectively). For the women who had a TVU, the ovarian follicle count was positively associated with AMH (Spearman correlation coefficient 0.694, p<0.001), and serum testosterone and androstenedione (0.338 p<0.001 and 0.411 p<0.001, respectively). 10.4% of the women who had a TVU had NIH criteria PCOS and 19% had Rotterdam criteria PCOS, the difference due to 12 women with PCOM and hyperandrogenism and 2 with PCOM and menstrual dysfunction.

Conclusions: AMH is a biochemical indicator of the ovarian follicle count. Testosterone directly/indirectly stimulates AMH production during folliculogenesis, hence the positive associations between serum androgens and AMH. Therefore, serum AMH, serum testosterone and the ovarian follicle count all identify the same biological phenomenon (number of developing follicles) and are not independent. Consequently, using PCOM and hyperandrogenemia to diagnose PCOS is effectively diagnosing PCOS using two dependent indices. Our data, together with the published literature, supports consideration of a revised PCOS criteria comprising menstrual dysfunction with either PCOM/elevated AMH or hyperandrogenism, excluding diagnosis based on PCOM plus hyperandrogenism.

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Association of gastric emptying time with gut hormones and appetite in Prader-Willi syndrome.

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Introduction:

Insatiable appetite, with uncontrolled hyperphagia, poses a challenging issue in Prader-Willi Syndrome (PWS). The causative factors of these abnormal eating behaviors remain unknown. It has been suggested that it may be caused by impaired hormone release which impacts the speed of gastric emptying (GE), and a change in hunger and fullness sensations.

Aims: To determine whether gut hormone levels have an impact on gastric emptying and/or appetite sensations in individuals with PWS, obese/overweight and lean controls.

Methods:

This is a observational cohort study where all subjects – PWS, obese/weight-matched and lean controls had a GE assessment by gastric scintigraphy. After eating a 99mTc-labelled breakfast (486kCal), images are acquired immediately after the meal and 1, 2 and 4 hours thereafter. Appetite sensations were assessed with a self-reported visual analog scale technique pre-meal and at specific time intervals post-meal with bloods drawn regularly to assess post prandial gut hormone release.

Results: 11 lean, 9 obese/weight-matched controls and 13 PWS subjects aged between 18 -51 were recruited. PWS had a similar average GE rate compared to lean and obese controls, however, 3 PWS subjects had delayed GE. There was no correlation observed between GE time and hunger and satiety scoring in all 3 groups. Similarly, we found no correlation between gastric emptying and appetite hormones in PWS and Obese subjects. In lean subjects, we observed a significant correlation between gastric emptying time and glucagon levels at 1 hour and 2 hours postprandially but not at 4 hours.

Conclusion: We identified 23% PWS subjects as having delayed GE, whereas GE was normal in all controls. Altered gut hormone levels doesn't seem to cause delayed GE. PWS subjects with delayed GE had similar appetite sensations as those with normal GE. This explains why delayed GE is usually undiagnosed in PWS.

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Longitudinal changes in serum testosterone and sex hormone-binding globulin in men aged 40-69 years from the UK Biobank

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Background: Serum testosterone concentration declines and sex hormone-binding globulin (SHBG) rises during male ageing. However, whether these changes reflect physiological ageing or accumulation of age-related comorbidities remains uncertain.

Objective: We examined the longitudinal changes in serum testosterone and SHBG concentrations in middle-aged to older men from the U.K. Biobank, the concordance between baseline and follow-up values, and their relationships with concomitant changes in sociodemographic and lifestyle factors.

Methods: Immunoassay serum total testosterone (n=7,813) and SHBG (n=6,491) were measured at baseline (2006-2010) and follow-up (2012-2013). Bland-Altman analyses and concordance correlation of repeated hormone measurements were conducted. Associations of changes in hormone concentrations with lifestyle and medical factors were explored using Spearman's rank correlation and bivariate hexbin plots.

Results: Over 4.3 years follow-up, there was a negligible mean change in serum total testosterone concentration (±SE) of +0.06±0.03 nmol/L, whereas mean SHBG concentration increased by +3.7±0.12 nmol/L. Concordance between measurements were 0.67 (95% confidence interval [CI]: 0.66-0.69) for total testosterone and 0.83 (CI=0.82-0.84) for SHBG concentrations. Changes in serum testosterone correlated with changes in SHBG (Spearman's rank ρ=0.33, CI=0.30-0.35), and inversely with changes in BMI (ρ=-0.18, CI=-0.20 to -0.16), and waist circumference (ρ=-0.13, CI=-0.15 to -0.11).

Conclusions: In relatively healthy middle-aged to older men, there was no evidence of a decline in mean testosterone concentrations over time, although mean SHBG concentrations increased. Although there was negligible net change observed, concomitant changes in SHBG, BMI, and waist circumference explained some of the variation in repeat measurements of testosterone. The relative stability of total testosterone concentration during follow-up supports the concept that healthy men can preserve endogenous testosterone production during ageing. These findings facilitate future research investigating associations of baseline testosterone with prospective health outcomes in ageing men.

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Do activin A and endocrine disruptors interact to affect testis development?

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Activin A is a member of the TGF-beta superfamily that is widely produced and influences many aspects of growth and disease in different organs. In the testis, activin A is important for normal development, including cord formation, regulation of somatic and germ cell proliferation, and steroid production *in utero*. Its levels are prematurely elevated during pregnancies with pre-eclampsia, and we are investigating potential links between disrupted activin A/TGF-b superfamily activities and testicular pathologies such human male infertility and testicular cancer. Our studies of mice lacking activin A (*Inhba*$^{-/-}$) and the activin A inhibitor, inhibin alpha (*Inha*$^{-/-}$), at embryonic (E) day 13.5, E15.5 and birth led us to conclude that activin A levels determine the ratio of germ to Sertoli (niche) cells. As a result, conditions of altered activin A in these mouse strains lead to altered testis cord shapes at birth. In *Inha* KO testes (elevated activin A levels), our histological studies identified germ cell phenotypes at E15.5 that are also observed following either *in vitro* or *in vivo* exposure to the endocrine disrupting chemical, di(2-ethylhexyl) phthalate (DEHP), or its metabolite, mono(2-ethylhexyl) phthalate (MEHP). Using a combination of RNA sequencing to interrogate testis transcriptomes in these mouse models, and testis cultures to examine acute exposure to either activin A and MEHP, we identified common and distinct effects. Culturing with both activin A plus MEHP resulted in an exaggerated phenotype, indicating that both influence similar processes. We discovered that activin A governs transcription of key genes involved in steroid production, affecting both Sertoli and Leydig cell function to control levels of testosterone and other steroids measured within the fetal testis. These results provide evidence that the outcomes of disruptions to activin/TGFbeta signalling during embryonic testis development may mimic or be exacerbated by exposures to endocrine disrupting chemicals.

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The Impact of Environmental Toxicants on Female Fertility

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Atrazine is one of the most widely used pesticides in the world, with 34,500 tonnes sprayed each year in the USA, and is frequently detected as a contaminant of ground, surface and drinking water. Each year in Australia, over 3,000 tonnes of atrazine is used in agriculture, on golf courses, and in suburban gardens. It is so pervasive in the environment that Australian water authorities actively monitor its concentration. Accumulating evidence in a number of vertebrate species suggests that atrazine impairs reproductive processes and can have significant effects on the health of subsequent generations. Whilst compelling, these studies have typically used short-term, high-dose exposure paradigms and have predominantly focussed on reproductive and health outcomes in adult males. In striking contrast, the impact of environmentally relevant exposures on ovarian function and female fertility, as well as multigenerational effects, remain poorly characterised. Therefore, using a combination of eutherian (mouse) and marsupial (dunnart) animal models, we are defining how exposure to atrazine, as an example of a well-defined environmental toxicant, affects female fertility and health across multiple generations. These studies will provide vital information about how synthetic toxicants like atrazine, which are highly pervasive and exponentially growing in our environment, affect the fertility and health of women, livestock and native mammals.

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The influence of prenatal exposure to endocrine disrupting chemicals on adult health

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Endocrine disrupting chemicals are ubiquitous environmental chemicals that influence the action of hormones. They may alter the expression, action, carriage within the blood, down-stream action, excretion and metabolism of hormones and consequently may have long term consequences. They gain access to the body through swallowing, breathing, through the skin, in breast milk and via the placenta. This talk will focus on prenatal exposures to endocrine disrupting chemicals that may lead to an influence on adult health, with a focus on my work in the field of reproduction.

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Comparison of Fracture Rates and Economic Outcomes by Age Group in Patients with Osteoporosis treated with Risedronate Enteric-Coated versus Immediate Release Bisphosphonates: A claims Data Analysis

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Objective: Risedronate Enteric-Coated (EC) is the only oral-bisphosphonate which can be taken with food, while preserving a higher bioavailability compared to immediate-release risedronate. Whether this formulation can further reduce the risk of fracture when compared to other Immediate-Release Bisphosphonate (IRB) remains unclear. Thus this study compared fracture rates and economic outcomes between women with osteoporosis treated with EC vs. IRB.

Methods: Women with osteoporosis were selected from a large US claims database (2009-2019). Patients were classified into EC or IRB cohorts based on the treatment initiated on the index date (first dispensing date for an oral bisphosphonate), matched 1:1 based on demographic and clinical characteristics, and observed for ≥2 years. Incidence rates (IRs) of fractures and healthcare resource utilization per 1,000 patient-years were compared between cohorts using IR ratios (IRRs). Outcomes were assessed overall and by age-groups (<65 yrs, ≥65 yrs and ≥75 yrs).

Results: Cohorts (n=2,726, median age: 60.0 yrs) were observed on average 4.5 yrs. The IR of fractures was significantly lower in the EC vs. the IRB cohort for any fracture site (EC: 34.65, IRB: 42.13; IRR=0.83, p<0.05) and spine fractures (EC: 10.84, IRB: 15.13; IRR=0.71, p<0.05). When stratified by age-group results persisted (table). Across the observation period, the IR of fracture was lower in the EC vs. the IRB cohort, reaching statistical significance at 36-months (fracture rate; EC=7.08%; IRB=8.67%, p=0.04). IR of hospitalizations was lower in the EC vs. the IRB cohort (EC: 106.74, IRB: 124.20; IRR=0.86, p<0.05) leading to significantly lower hospitalization costs among EC patients (average per-patient-per-year; EC: US$3,611; IRB: US$4,603, p<0.05).

Conclusion: Women with osteoporosis treated with EC have a lower incidence of fracture when compared to IRB. Potentially indicating that the bioavailability and therefore the efficacy of EC is higher than IRB, independent of food intake.

Table: Compares the Incidence Rates (IR) and IR ratios (IRR) of any fracture site stratified by age-group

	N	IR		IRR (95% CI)
		EC	IRB	
All	2,726	34.65	42.13	0.83 (0.70 – 0.97) *
< 65 Yrs	1,896	23.69	24.37	0.96 (0.75 - 1.24)
≥ 65 Yrs	830	58.65	80.79	0.74 (0.60 - 0.91) *
≥ 75 Yrs	392	81.22	102.84	0.80 (0.61 - 1.05)

**p<0.05, EC* = Risedronate Enteric-Coated, IRB = Immediate-Release Bisphosphonate

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Health outcomes in elderly individuals with acute severely painful osteoporotic vertebral compression fractures

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Background: There are minimal data describing outcomes after acute clinical osteoporotic vertebral compression fractures (OVCF).

Aim: We report on pain scores, quality of life measures and complication and specified osteofragility risk factors in the placebo-treated individuals in the VAPOUR study.

Methods: VAPOUR is a multi-centre randomised, blinded, parallel group, placebo-controlled trial of vertebroplasty for painful OVCF performed within 6-weeks post fracture. The entry criteria were patients' age > 60, back pain < 6 weeks duration, numeric rated scale (NRS) pain ≥ 7/10, and Magnetic Resonance Imaging (MRI) or single-photon emission computed tomography confirming one or two recent fractures. Primary outcome measure was numeric rated scale (NRS) pain, on a scale of 0-10 and the secondary outcome measure was Roland-Morris Disability Questionnaire (RMQ).

Results: Data were available on 59 individuals, mean age of 81years, 68% were female and 86% were receiving anti-osteoporotic therapies. 42% of individuals were treated as outpatient and 58% were hospitalised. 46% had pre-existing OVCF. 66% had Genant grade 3 deformities in the newly diagnosed vertebral fracture. The mean NRS pain (8.6) and RMQ scores (19.8) was indicative of severe pain and loss of function on presentation. By 6 months, three individuals (5%) withdrew, three (5%) had died, two (4%) had developed spinal cord compression and two (5%) had sustained new OVCF's. Mean baseline fracture compression of 46% of height loss had increased to 63%. Despite overall improvements, 27 (53%) had ongoing problematic back pain (numeric rating scale ≥4) and 32 (63%) had significant disability (Roland Morris Questionnaire ≥10) at 6-months. 44 (75%) were considered high risk for recurrent OVCF.

Conclusion: These data confirm that elderly individuals with acute severely painful OVCF treated with usual care, demonstrate adverse outcomes even at 6 months after fracture. Further research should focus on optimal treatments for this high-risk cohort.

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Skeletal Age: a new score for fracture risk assessment

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Many fragility fractures are associated with reduced life expectancy, which is usually not part of the doctor-patient discussion about fracture risk. We propose a new score called "Skeletal Age" to define the relationship between fracture and mortality for such a discussion. This study sought to estimate Skeletal Age based on risk factors.

Skeletal Age was defined as the age of an individual's skeleton resulting from a fragility fracture. Thus, for an individual with a fracture associated with increased mortality risk, the Skeletal Age would be expected to be higher than the individual's chronological age. We first used the Gompertz law of mortality to transform the hazard ratio of mortality for each fracture site into life expectancy as a result of a fracture. The difference between life expectancy associated with a fracture and population life expectancy is the loss of life years. Skeletal Age is then operationally defined as an individual's current age plus the years of life expectancy lost. This study used the Danish nationwide registry-based data including all individuals aged 50+ years.

During a median follow-up of 14.1 years (IQR:5.5-16.0), 95,372 men and 212,498 women sustained an incident fracture followed by 41,017 and 81,727 deaths, respectively. On average, a fragility fracture was associated with 1 to 3 years of life lost, with greater loss being observed in men and younger patients. Hip and proximal fractures, but not distal fractures, were associated with a substantial loss in life expectancy. For example, 60-year man with a hip fracture was calculated as having a skeletal age of 64.5 years (i.e., 4.5 years of life lost) (Table).

We propose that the Skeletal Age replaces relative risk as a score for conveying the risk of mortality associated with a fragility fracture, and for communicating the effect of fracture on life expectancy to patients.

Table. Skeletal Age for a 60-year old patient with a fracture associated with increased mortality risk

Fracture	Men	Women
Any fragility fracture	62.8 (62.7, 62.9)	61.9 (61.8, 62.0)
Specific fracture sites:		
Hip	64.5 (64.3, 64.6)	63.5 (63.4, 63.6)
Femur	64.2 (63.7, 64.6)	63.7 (63.4, 64.1)
Pelvis	64.0 (63.4, 64.5)	63.2 (62.9, 63.5)
Vertebrae	63.3 (63.0, 63.6)	63.2 (63.0, 63.4)
Humerus	63.7 (63.4, 63.9)	61.7 (61.6, 61.9)
Rib	62.1 (61.7, 62.4)	62.2 (61.8, 62.6)
Clavicle	62.4 (62.0, 62.8)	62.3 (61.9, 62.7)
Lower leg	61.6 (61.2, 61.9)	61.4 (61.1, 61.7)

Data presented as years (95% CI).

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The Sydney AFF Score: A Simple Tool to Distinguish Females Presenting with Atypical Femur Fractures versus Typical Femur Fractures

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Purpose

Atypical femur fractures (AFF) are a rare but serious complication of long-term bisphosphonate use. Although clearly defined by American Society for Bone and Mineral Research (ASBMR) criteria, the use of qualitative fracture criteria may lead to uncertainty in AFF diagnosis, with significant therapeutic implications. A score that rapidly and accurately identifies AFFs amongst typical femur fractures using quantitative parameters is needed.

Methodology

Radiographs of femoral shaft and subtrochanteric fractures treated at a tertiary centre in Sydney from January 2008 - May 2017 were retrieved using Electronic Medical Record coding. Subsequently, 413 anteroposterior pelvic radiographs with morphological characteristics of AFFs were reviewed by three expert adjudicators and classified as AFFs or non-AFFs. Geometric and demographic data was analysed with multiple logistic regression and decision tree analysis to develop the Sydney AFF risk score. This score was validated on a patient population in an separate ethnically diverse tertiary centre.

Results

This score uses three dichotomised independent variables and adds one point for each: [age £ 80 years] + [femoral neck width <37 mm] + [lateral cortical width at lesser trochanter ³5 mm], (score 0 – 3). In an independent set of 53 female patients at a different centre in Sydney, a score ³2 demonstrated 73.3% sensitivity and 69.6% specificity for AFF (AUC 0.775, SE 0.063) and remained independently associated with AFF after adjustment for bisphosphonate use. Within the AFF population, distinctions in femoral geometry were evident in patients of Asian ethnicity.

Conclusion

The Sydney AFF Score provides a quantitative means of identifying female patients with femur fractures who have sustained an AFF as opposed to a TFF. This score has clear management implications and this score may augment ASBMR diagnostic criteria.

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Misalignment by producing Ineffective Load Conduction May be Responsible for Atypical Femoral Fractures (AFFs)

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Introduction –The lack of a tool to identify individuals at risk for AFFs is an important unmet need in osteoporosis management.

Bone has two key different mechanical functions: (i) to bear loads, this requires high bone density and strength; (**ii.)** to conduct (transmit) received forces (loads) until they reach a final structure. (e.g., sound conduction by the middle ear ossicles). To perform this second function effectively, bone does not need to be dense or strong. Instead, bone needs to be a good conductor to efficiently receive and transmit loads. To achieve this, an effective cohesion between components is required- i.e., **good alignment.**

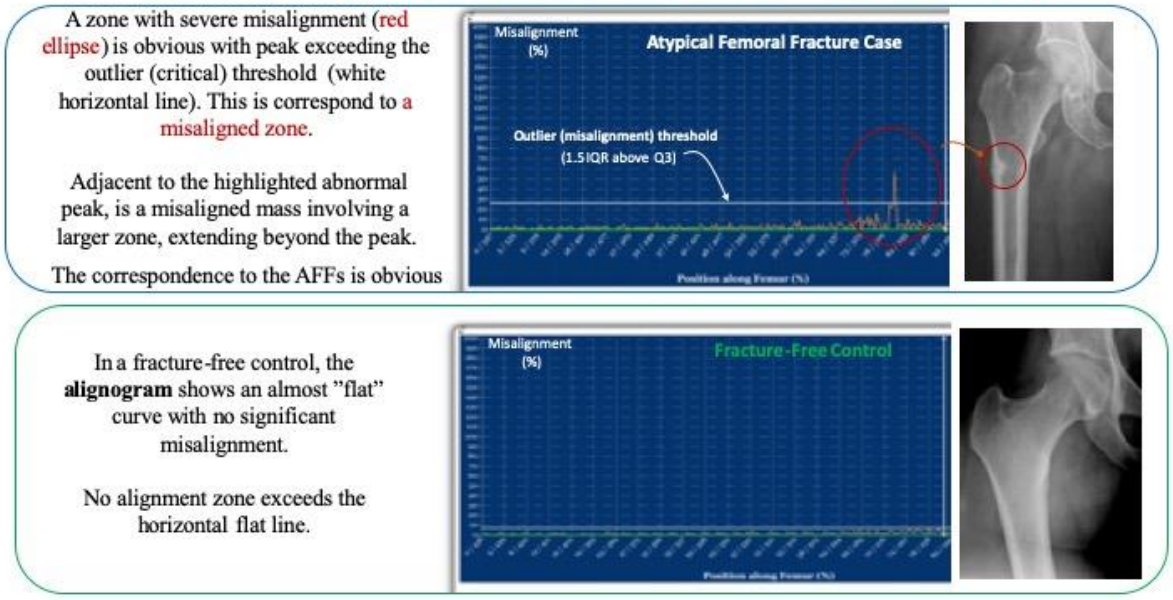
Impairment in either function may produce fracture. As AFFs are localized, associated with deformities (e.g. bowing) and occur in settings of relatively preserved bone density, we propose ineffective load transfer produced by misalignment is an important mechanism responsible for AFFs. Thus, comparing AFFs (**n=4**) and fracture-free controls (**n=6**), we tested the hypothesis that femoral misalignment is associated with AFFs.

Methods – Accordingly, we develop a ***Misalignment Detector*** (**Alignogram**$_{1.0}$); Using Pelvic X-rays, this novel software quantifies and displays the degree of misalignment (expressed as percent) at each position along the femur as a curve. The **Alignogram** further separates the curve into zones, and highlights misaligned zones (outliers).

Results – AFFs patients had a 4-fold greater misalignment than controls (4.8±0.96 vs 19.18±2.25 %;p<0.001); and a more heterogeneous curve (1.61±0.23 vs 2.78±0.20;p=0.0007). In all patients with AFFs, the **Alignogram** showed at least one misaligned zone (**see figure**). None was found in controls.

Conclusion – AFFs are associated with ineffective load transfer rather than reduced bone density or strength. Measurement of misalignment from readily available *X-ray* images may hold the key in identifying patients at risk for AFFs. Thus, guiding appropriate therapeutic decisions. Larger studies are needed to confirm our findings.

Figure Examples of **Alignograms** displaying the magnitude of misalignment (*y-axis*) at each position along the femur (*x-axis*) in a patient with Atypical Femoral Fracture (AFFs) *versus* a Fracture–free control



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Romosozumab (Romo) treatment lowers the incidence of new vertebral fractures (Vfx) across all fracture severity grades among women with postmenopausal osteoporosis (PMO)

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We assessed the incidence of new VFx by Genant severity grade in the Romo vs placebo (Pbo) or alendronate (ALN) arms of the FRAME and ARCH studies, respectively.

The incidence of new VFx was significantly lower among patients who received Romo during the 12-month double-blind treatment phase in both studies. Over 12 months, the incidence of new VFx was 0.5% Romo vs 1.8% Pbo (P<0.001) in FRAME and 3.2% Romo vs 5.0% ALN (P=0.008) in ARCH. Over 24 months, the incidence of new VFx was 0.6% Romo→DMAb vs 2.5% Pbo→DMAb (P<0.001) in FRAME and 4.1% Romo→ALN vs 8.0% ALN→ALN (P<0.001) in ARCH. Fewer new VFx were observed in the Romo arm of both studies across all fracture severity grades. Specifically, in FRAME, the incidence of mild VFx was 0.2% Romo vs 0.4% Pbo over 12 months and 0.2% Romo→DMAb vs 0.6% Pbo→DMAb over 24 months; the incidence of moderate VFx was 0.1% Romo vs 0.9% Pbo over 12 months and 0.2% Romo→DMAb vs 1.4% Pbo→DMAb over 24 months; and the incidence of severe VFx was 0.2% Romo vs 0.5% Pbo over 12 months and 0.2% Romo→DMAb vs 0.6% Pbo→DMAb over 24 months. Similarly, in ARCH, the incidence of mild VFx was 0.5% Romo vs 1.0% ALN over 12 months and 0.4% Romo→ALN vs 1.4% ALN→ALN over 24 months; the incidence of moderate VFx was 1.3% Romo vs 2.1% ALN over 12 months and 1.8% Romo→ALN vs 3.4% ALN→ALN over 24 months; and the incidence of severe VFx was 1.5% Romo vs 1.9% ALN over 12 months and 1.9% Romo→ALN vs 3.3% ALN→ALN over 24 months.

In conclusion, Romo administered over 12 months resulted in reductions in VFx across all fracture severity grades compared with Pbo and ALN; the treatment effect continued after patients transitioned to an antiresorptive.

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Tricky Cases in Bone Disease

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Available Soon

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Adrenarche - Searching for Mechanistic Understanding of Cause and Consequence.

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Adrenarche is the prepubertal increase in synthesis and secretion of the androgen dehydroepiandrosterone (DHEA) from the zona reticularis (ZR) of the adrenal gland. It is widely considered to be an early-life phenomenon unique to humans and only some non-human primates. The functional consequences of the adrenarche for childhood brain development are not fully understood; ethical restrictions preventing experiments in humans and young primates limits our mechanistic understanding of the adrenarche and its significance. The factors driving the developmental change in ZR androgen activity have not been fully identified; they may be intra-adrenal, extra-adrenal, or both. While discussion of the adrenarche focusses attention on the pre-pubertal surge of DHEA/DHEAS, these steroids are in fact dynamically regulated from before birth, and therefore may be regulated by factors affecting fetal growth, the placenta, the hypothalamic-pituitary axis, or indeed the spino-sympathetic innervation of the adrenal medulla and ZR. In addition, it is now known that DHEA/DHEAS is synthesized in the brain during development in some species, but it is not known if this is co-ordinated with adrenal production of this androgen. If DHEA/DHEAS is indeed important for brain maturation both before and after birth, and important for the adaptation and rewiring of the postnatal brain to the new and ever-changing challenges of real-world and social life, we suggest that the difficulty of investigating these neurodevelopmental phenomena in humans and a few primates could be resolved by looking for new animal models in which adrenarche-like changes occur during pre- and post-natal development.

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Clinical, Biochemical and Histological Caveats in the Assessment of Primary Aldosteronism

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Primary aldosteronism (PA) is the most common endocrine cause of hypertension that affects ~5-10% of hypertensive patients in the community. Current recommendations for screening may lead to missed opportunity for diagnosis based on emerging evidence from studies in primary care. Following screening with the aldosterone to renin ratio, the interpretation of confirmatory test results needs to consider aldosterone assay characteristics as well as sample handling and storage. The classification of PA as unilateral or bilateral subtypes is based on adrenal imaging and adrenal vein sampling. The success of adrenal vein cannulation and the degree of lateralisation of aldosterone excess can be significantly affected by intra-procedural sedation and ACTH stimulation. In patients with unilateral disease who undergo adrenalectomy, their adrenal histopathology is no longer the simple distinction between adenoma and hyperplasia. CYP11B2 staining is a new player with an impact on clinical outcomes.

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'Relative Contributions of De Novo Synthesised and Recycled Adrenal Glucocorticoids' (Zone Fasciculata)

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Glucocorticoids were traditionally thought to exert their effects through direct actions of adrenally synthesised steroids on target tissues. However, it is now clear that a proportion of glucocorticoid action at a tissue level is through local reactivation of inactive precursors via the 11b-hydroxysteroid dehydrogenase type 1 enzyme (11b-HSD1). 11b-HSD1 converts inactive glucocorticoids such as cortisone (human) and dehydrocorticosterone (DHC) to their active counterparts cortisol and corticosterone. The enzyme is also essential for the conversion of prednisone (inactive) to prednisolone (active). In both normal physiology and in states of glucocorticoid excess the relative contributions of circulating active and locally reactivated glucocorticoids to (patho)physiology is unclear but there is increasing data to suggest that local reactivation is of critical importance in various situations.

In an early human clinical study the effects of prednisolone on bone were predicted by an individuals 11b-HSD1 activity rather than the level of active drug in the circulation. Additionally, 11b-HSD1 knockout mice appear strongly protected against the effect of glucocorticoid excess on metabolic parameters again indicating an important role for indirect reactivation of glucocorticoids.

We have further examined this issue in relation to inflammation in knockout models of murine joint and muscle inflammation with and without treatment with therapeutic glucocorticoids. In these models the immunosuppressive effects of glucocorticoids appear to be mediated primarily via 11b-HSD1 activity and the consequent regeneration of inactive to active glucocorticoids. Our current studies examining healing after burn injury demonstrate that application of active and inactive glucocorticoids influences the healing process but that inactive glucocorticoids have a greater ability to optimise wound outcomes that active glucocorticoids.

The realisation that glucocorticoids can exert their actions through direct and indirect routes creates opportunities to separate out these functions and enables novel therapeutic strategies to be employed.

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Nuclear Imaging and Radionuclide Therapy for Phaeochromocytoma and Paragangliomas (PPGL)

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Phaeochromocytoma and Paraganglioma (PPGL) are relatively uncommon and clinically heterogenous neuroendocrine tumors that arise from pluripotent neural crest cells. Treatment options for unresectable or metastatic PPGL, especially for patients with uncontrolled secondary hypertension, are limited. Oncologic radionuclide treatment conventionally included the use of 131I-metaiodobenzylguanidine (MIBG) therapy for tumors demonstrating high uptake on MIBG imaging. Recently, peptide receptor radionuclide therapy (PRRT) using 177Lu or 90Y-labelled peptides have shown encouraging responses for PPGLs with high somatostatin receptor expression detected on 68Ga-labeled-peptide PET/CT imaging, as a promising option and potential alternative to MIBG theranostics. This session will provide an overview of nuclear imaging and radionuclide therapy available for patients with metastatic PPGL focusing on MIBG and somatostatin receptor targeting. We will review the current evidence, discuss its oncologic applications with case examples, address practical issues and outline potential emerging personalized approaches in patients with metastatic PPGL.

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Beneath the Cartilage: How Bone Tissue Alterations Contribute to Osteoarthritis Progression

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Osteoarthritis (OA) is an increasingly prevalent age-related joint disease with a high burden of personal and economic cost. The current failure to understand the underlying mechanisms of OA has prevented the development of effective disease modifying treatments. OA is a whole-joint disease, in which all components of the joint are affected, with particular involvement of the articular cartilage and subchondral bone. The disease is characterised by articular cartilage degeneration, with the addition of both generalised and focal changes of the subchondral bone. Bone marrow lesions (BMLs) are localised MRI features that are frequently found in the subchondral bone of patients with both early and late stage of OA. BMLs associate strongly with joint pain, structural degeneration of the articular cartilage, and predict progression to joint replacement. Our recent work in human knee OA has involved detailed histopathological examination of the osteochondral tissue that comprises BMLs. BML tissue is characterised by severe overlying cartilage degeneration and significant bone changes, which include increased trabecular bone volume with associated low bone matrix mineralisation, and increased microdamage (i.e. microcrack burden), vascularity and bone remodelling. Also, BML presence is shown to predict more extensive subchondral bone microstructural changes in the human OA knee. From this work, BMLs appear to represent a focal 'hot zone' of bone remodelling activity and may be the initiating epicentre of structural changes in the OA joint. The data support the use of BMLs as MRI image-based biomarkers to inform on the degenerative state within the OA knee. This presentation will review the role of subchondral bone focal changes in the initiation, development, and progression of OA, with an emphasis on BMLs in human OA and discussion of new data linking localised subchondral bone TGF-beta activity to impaired bone quality and the severity of human OA.

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Therapeutic Strategies for Osteoarthritis: Lessons Learned from Failed Clinical Trials

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Osteoarthritis (OA) is a highly prevalent, painful, disabling, and costly condition. Our understanding of OA has dramatically improved over the course of the 20th century, and it is now recognised as a whole-joint disease. Although its signature pathologic feature is articular cartilage loss, it commonly involves many other joint structures including subchondral bone, ligaments, menisci, muscles, peripheral nerves, and synovium.

Despite OA's large disease burden there are currently no approved disease-modifying OA drugs (DMOADs) that can prevent or delay the progression of the disease. Current Food and Drug Administration (FDA)-approved treatments help to reduce symptoms but do not prevent ongoing joint structural damage. The overall lack of treatment efficacy may be partly due to a 'one-size-fits-all' treatment approach. OA has proven to be a more complex, heterogeneous disease than was originally thought and may require different approaches for each patient to optimise treatment. It is not only a disease of cartilage but can be divided into multiple phenotypes (e.g., bone-, inflammatory-, and cartilage- phenotypes). To some extent, these phenotypes overlap with one another. By targeting the most actively affected joint tissue in a patient, during a particular phase of disease, it may be possible to identify more effective treatments.

Bisphosphonates are a class of drugs that have been considered a promising candidate to treat bony phenotypes of OA. In a proof-of-principle study we demonstrated that zoledronic acid (a intravenous bisphosphonate) reduced knee pain and size of subchondral bone marrow lesions (BMLs) over 6 months in knee OA patients with a bony phenotype. This talk will present the findings from a larger, multicentre, double-blind, placebo-controlled trial which examined the effect of zoledronic acid on knee cartilage loss over 24-months. It will discuss the challenges faced for identifying new drug targets for osteoarthritis and possible steps to overcome these barriers

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Sex-specific Changes in the Placenta Associated with Prenatal Alcohol Exposure

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Many women of reproductive age consume alcohol, often at binge levels. Coupled with high rates of unplanned pregnancy (~50%), exposure of the early embryo to prenatal alcohol is inevitable in many pregnancies. Additionally, ~40% of Australian women continue to consume alcohol throughout pregnancy, albeit at lower levels. Impacts on the development, morphology and function of the placenta likely underlie the increased risk of pregnancy complications and long-term adverse outcomes associated with prenatal alcohol exposure. Typically, adaptations by the placenta to pregnancy complications are sex-specific.

Our recent systematic review and meta-analysis, across 33 included clinical studies, has shown that prenatal alcohol exposure increases the likelihood of placental abruption and is associated with decreased placental weight; and altered placental vasculature, DNA methylation and molecular pathways. However, only a single study examined placentas by fetal sex, confirming sex-specific outcomes.

We have utilised our unique rat model of periconceptional ethanol exposure, from 4 days prior to mating until embryonic-day 4 (E4), to further explore potential sex-specific changes in the placenta. The periconceptional period is a critical time when exposure to an adverse environment can impact the pregnancy and program long-term disease in offspring. This model results in female-specific alterations in trophoblast outgrowth capacity in the early embryo at E5, as well as reduced maternal blood space volume in the placenta at E15. There is also evidence of global hypermethylation in the blastocyst, indicative of inappropriate epigenetic reprogramming, along with reduced maternal plasma choline levels and altered components of the one-carbon metabolism pathway in the E20 placenta. Placental efficiency was reduced and there was increased accumulation of glycogen in late gestation female placentas. Our results highlight that alcohol should not be consumed by women during pregnancy or when planning a pregnancy, and that public health campaigns should target prevention prior to conception.

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Selenium and Selenoproteins at the Feto-Maternal Interface

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Selenium is an essential trace element and low selenium status has been associated with poor gestational outcomes. Selenium is metabolised to the amino acids Selenomethionine and selenocysteine and the latter forms the active site of some 25 selenoproteins. The majority of these can be found in the human placenta. Glutathione Peroxidases (GPx) and Thioredoxin reductases (ThxRed) have been extensively studied in the human placenta and play a role in regulating redox homeostasis. Placental oxidative stress at the mitochondrial/ER interface is increased in preeclampsia, fetal growth restriction and some cases of preterm birth. Decreased expression and specific cellular localisation of these selenoenzymes has been implicated in the development of placental oxidative stress. Whilst we know much about the important role of GPx and ThxRed in the placenta less is known of the role that other selenoproteins may play at the feto-maternal interface. This presentation will review the current data on selenium status and gestational outcomes, the importance of placental selenoproteins and propose a critical role for selected selenoproteins in responding to stress at the mitochondrial/ER interface in the human placenta.

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Convergent evolution of the vertebrate placenta

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Pregnancy is an important biological innovation that has evolved convergently hundreds of times in fish, mammals, reptiles, amphibians, and invertebrates. While many viviparous lineages nourish developing embryos from egg yolk alone, complex placentae that transport large quantities of organic nutrients to the fetus (known as placentotrophy) have evolved independently at least seven times in amniotes (at least once in therian mammals and six times in scincid lizards). The placenta has arisen via homologous associations of the same extraembryonic membranes and uterine tissues in each lineage. Analogous placentae have also evolved multiple times in sharks and teleost fish (anamniotes), but from different ancestral structures, because their embryos lack the amniote-specific extraembryonic membranes. We are currently combining morphological, transcriptomic and

proteomic approaches to determine how placentae support embryonic development in lizards, sharks, and mammals. This work is shedding light on the fundamental biology of the placenta in poorly studied species, for example, in determining the mechanisms underpinning nutrient transport in a placental shark that allow embryonic mass increase of almost 3,000 % over 4.5 months of development. Our research is also contributing to new knowledge in evolutionary biology, as we determine whether the same genes have been recruited to support placental function across diverse animals.

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Getting to heart of it: the placental-cardiovascular axis

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Diminished placental vasculature, particularly on the fetal side of the placental vasculature, associates with fetal growth restriction. Aberrant feto-placental vascular structure impacts fetal growth but less clear are the implications for fetal cardiovascular development. Loading of the fetal heart is determined by incoming placental flow, and furthermore the fetal heart beats directly against resistance of the placental vascular bed. Thus placental haemodynamics likely have important influences on cardiac development. Moreover, in FGR, regional blood flow can change, with a higher proportion of blood coming from the placenta bypassing the fetal liver with increased perfusion of the fetal head and neck. These haemodynamic shifts impact fetal cardiovascular development, with both short and long-term health implications. To explore this placental-cardiovascular axis, we have utilised a range of models of glucocorticoid exposure in pregnancy. Glucocorticoids are critical for fetal maturation, including the heart, and in excess are known to reduce feto-placental vasculature and fetal growth. We have established that glucocorticoid receptor signalling is essential for gestational maturation in diastolic flow within the umbilical artery and that acute exposure to glucocorticoids in mouse pregnancy has gestation-dependent effects on fetal and placental haemodynamics. Utilising a rat model of chronic glucocorticoid exposure we have made significant advances in imaging and modelling the longitudinal haemodynamics of the feto-placental cardiovascular axis. These approaches will drive the development of more accurate diagnostic criteria for at-risk pregnancies, as well as the prediction of offspring health complications and consideration of preventative strategies.

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Deriving induced pluripotent stem cells (iPSCs) for the Fat-tailed dunnart

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Marsupial models have so much to teach us about fundamental mammalian biology. However, essential technologies that are well defined for conventional laboratory models are concerningly underdeveloped for our marsupials. Stem cells are an integral tool for studying developmental biology and genetics. Unfortunately, the absence of an inner cell mass in marsupial embryos prevents the isolation of embryonic stem cells. Instead, we focused on the generation of induced pluripotent stem cells (iPSCs) as an equivalent alternative, using the Fat tailed dunnart as a model. iPSCs are produced by transfecting somatic cells with pluripotency genes in a process known as reprogramming. The induction of reprogramming is controlled by a highly conserved set of core factors. However, the conditions required for its completion are usually species specific, requiring additional genes, small molecules, specialised culture conditions or other variations in the derivation protocol. I have tested combinations of reprogramming plasmids, media and several small molecule supplements to refine conditions for reproducibly producing iPSCs from dunnart fibroblasts. This protocol has now been used to produce two distinct lines and multiple clones. Cells show high expression of pluripotency markers as validated using qPCR and immunocytochemistry. In concordance with other marsupial work, higher expression of POU5F3 relative to OCT3/4 has been observed. Embryoid bodies have been produced for both lines in both suspension and adhesion conditions. These have been shown to differentiate into non-stem lineages. Single cell gene expression libraries have been produced and sequenced, making these cells an important resource for marsupial pluripotency. iPSC technology makes the dunnart a tractable model for identifying conserved regulatory pathways in mammalian development. The use of stem cells should also have vast implications for marsupial conservation efforts.

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Divergent gonadal cell origin and lineage specification in amniotes revealed by single-cell transcriptomics.

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Due to the process of gonadal sex determination, the key supporting cell lineage differentiates into Sertoli cells in the testis or pre-granulosa cells in the ovary. Supporting cells are derived from a single sexually bipotential precursor lineage present in the early gonadal primordium. In mammals, it has been shown that the supporting cell progenitor line develops via ingression and EMT of cells from the coelomic epithelium. While the genetic triggers for gonadal sex differentiation vary across species, the cell biology of gonadal development was long thought to be largely conserved. Here, we present a comprehensive analysis of gonadal sex differentiation, using single-cell RNA sequencing in the embryonic chicken gonad during sexual differentiation. Combining lineage tracing with single cell transcriptomics, the data show that somatic supporting cells of the embryonic chicken gonad do not derive from the coelomic epithelium, in contrast to other vertebrates studied (mouse and turtle). Instead, the early somatic precursors cells of the gonads in both sexes derive from a resident *DMRT1+/PAX2+/WNT4+/OSR1+* mesenchymal cell population. *PAX2* was identified as a novel undifferentiated supporting cell marker in chicken, being downregulated at embryonic day (E) 6.0, consistent with the upregulation of *DMRT1* expression in males and testicular differentiation. We were able to confirm

PAX2 positive cells in quail, emu and zebra finch undifferentiated gonads, but not in mouse nor the bearded dragon (*Pogona vitticeps*). This suggests that the PAX2+ mesenchymal origin of supporting cells is conserved among birds, but not in reptiles or mammals. Altogether, these results indicate that, just as the genetic trigger for sex differs across vertebrate groups, cell lineage specification in the gonad may also vary substantially.

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Engineering anti-Müllerian hormone (AMH) analogues to preserve female fertility

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Within the ovary, anti-Müllerian hormone (AMH) is secreted by the granulosa cells of small growing follicles, and limits the activation of primordial follicles. In a series of studies, it has been shown that supraphysiological levels of AMH can (i) act as a contraceptive, blocking folliculogenesis at the primary stage, and (ii) safeguard the ovary from chemotherapy-induced fertility insults. As such, AMH technologies are attractive tools for the preservation of female fertility. However, the development of AMH technologies is currently limited by both an incomplete understanding of the mechanisms of AMH action, and an inability to manufacture sufficient amounts of AMH for in vivo delivery. Here, we first aimed to improve the production of bioactive AMH. To address this, we used targeted mutagenesis to improve the processing efficiency of pro-AMH. Substitution of the native cleavage site intervening the AMH pro- and mature domains with an ideal processing site increased the yield of mature AMH by up to 5-fold. Next, we aimed to enhance the potency of AMH by improving the ability of AMH to bind to its target receptors. To achieve this, we used targeted mutagenesis to identify loss and gain of function AMH mutants. Using AMH-responsive *in vitro* assays, we identified a key mutation in AMH that enhanced AMH bioactivity by as much as 5-fold. Excitingly, combining the cleavage site and receptor-binding modifications resulted in an overall 100-fold improvement in AMH bioactivity. These experiments also uncovered key residues in AMH that mediate interactions with the AMH receptors. Ultimately, these studies have improved our understanding of the mechanisms of AMH bioactivity, and enabled the generation of a more potent AMH analogue. Our AMH analogues are attractive tools for preserving female fertility, and also have applications as non-surgical sterility agents in animals.

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Biobanking reproductive tissue in threatened Australian fish species

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Many small, Australian, freshwater fish species are at risk of extinction within the next 20 years, meaning immediate and targeted efforts for conservation are required. An emerging tool in conservation is the cryopreservation and storage of reproductive cells and tissues in facilities known as “frozen zoos”. This approach creates a cellular “back-up” and could play a critical role in future conservation efforts if a species were to go extinct. Unfortunately, the cryopreservation of fish oocytes and embryos has faced significant challenges, limiting the use of gamete cryopreservation in threatened fish species. One alternative is the cryopreservation of gonadal tissue which contains early germ line cells, the spermatogonia and oogonia.

We describe the successful cryopreservation of testicular and ovarian tissue in *Melanotaenia fluviatilis,* from the family Melanotaeniidae (order: Atheriniformes) which contains several species listed as endangered or critically endangered on the IUCN Red List. Post-thaw viability of cells from cryopreserved gonadal tissue was comparative to fresh controls in both testis and ovarian tissue, with viabilities of 72.6%±10.5% and 63.5%±18.2%, respectively. Additional comparative experiments were conducted in *M. australis* which indicated our cryopreservation protocol could be applied across the *Melanotaenia* genus. Using flow cytometry, we also optimised a method to produce enriched samples of spermatogonia and oogonia from gonadal tissue. The isolation of gonial cells is critical for future downstream methods used to produce gametes from these cells such as cell surrogacy or *in vitro* differentiation.

We present the first protocol for the biobanking of reproductive tissue from members of Melanotaeniidae, and the order Atheriniformes, and in doing so, provide a framework for further investigation into the use of biobanking and assisted reproduction in the conservation of rainbowfish, and other Australian fish species.

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A novel in vitro differentiation protocol for human embryonic gonadal organoids as a disease model for differences of sex development.

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Differences of Sex Development affect an alarming 1.7% of babies and can be caused by aberrations in the genetic pathways that control embryonic gonadal development. Currently, less than 40% of children born with a 46,XY DSD receive a genetic diagnosis, leaving 60% with either a negative genetic finding or one of uncertain significance. To improve the diagnostic rate, functional validation of novel or uncertain genetic findings is required, something that is currently extremely challenging in DSD due to the lack of a human embryonic gonadal cell line, and the near impossibility of obtaining primary human embryonic gonadal tissue. Therefore, there is an urgent need for an in vitro model of human gonadal development to study these disorders. We have addressed this by establishing a world-first protocol to differentiate human induced pluripotent stem cells (iPSCs) into early gonadal cells cultured as testis-like organoids. Our stepwise differentiation protocol uses small molecules to mimic developmental signalling, inducing iPSCs to develop into the bipotential gonad by day 7. Aggregating and culturing these cells as 3D organoids results in testis gene expression in cells that reside within tube-like structures delineated by basement membrane, reminiscent of cord-like assemblies in re-aggregated mouse testes. Transcriptomic profiling of organoids using single cell RNA sequencing shows gonadal and reproductive tissue identities, and distinct testicular cell lineages which overlap with those found in human fetal gonads. Disease modelling using an iPSC line carrying a novel genetic mutation implicated in gonadal dysgenesis results in gonad organoids with reduced growth, altered structure and accelerated cell death. Here we discuss the ongoing work to expand this methodology to include additional more mature testis cell types, to produce an organoid that fully recapitulates the human fetal testis. This innovative disease model would bring about a paradigm shift in the study of fetal gonadal health.

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The effects of acute *in vivo* LHCGR stimulation on adult mouse Leydig cells

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Leydig cells produce androgens to support fertility and other androgen-dependent functions. At puberty, adult Leydig cells (ALCs) produce androgens upon activation of the LHCG receptor (LHCGR) by luteinising hormone (LH). Many Leydig cell genes have been shown to be responsive to LHCGR activation by LH or human chorionic gonadotrophin (hCG). Yet there has been no comprehensive evaluation of the effects of this activation on the mouse ALC transcriptome *in vivo*. We aimed to identify genes and pathways in ALCs altered by acute *in vivo* LHCGR stimulation via administration of hCG. Adult (100 days old) transgenic mice expressing *GFP-Nr5a1*[1] were injected with vehicle or hCG (5IU/30g body weight). Mice were sacrificed 2 or 6 hours later and testicular ALCs (GFP^{+++}) isolated by FACS to produce a highly purified population[1]. RNA was sequenced (Illumina HiSeq 2500, 50 bp, paired end, ~35 million reads), bioinformatic analyses performed in R (v3.3.1) and DESeq2 identified differentially expressed genes (DEGs, log2 fold change <-0.5 or >0.5 plus p adj<0.01). After 2hrs of LHCGR stimulation, 1363 transcripts were up-regulated and were significantly associated with cholesterol and steroid metabolism (indicative of stimulation of steroidogenesis) and cell-cell adhesion. In addition, 1524 transcripts were decreased and significantly associated with transcription and DNA damage/repair. Six hours of LHCGR stimulation induced 1297 genes that were highly enriched in functions associated with innate immunity and response to interferon-beta, whereas 1869 down-regulated genes were associated with mitochondria and lipid metabolism. Resident macrophages in the testis can enhance steroidogenesis[2], and our results suggest LHCGR activation may drive ALC-immune cell interactions *in vivo* that support optimal steroidogenesis. We also identified transcripts highly responsive to acute LHCGR stimulation, with unknown functions in Leydig cells, providing opportunities to discover novel regulatory pathways. These data provide new insights into how LHCGR activation supports optimal steroidogenesis and Leydig cell function.

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The developmental origins of mammographic density and breast cancer risk

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High mammographic density is an independent risk factor for breast cancer. Epidemiological studies show that high body mass index in puberty is associated with low adult mammographic density and reduced lifetime breast cancer risk. This suggests that pubertal adiposity affects adult breast health, however, causal mechanisms are yet to be elucidated. This project investigated whether pubertal adiposity is causal in mammary fibroglandular density and cancer development in adulthood using mouse models.

Alms1bbb/bbb mice overeat and exhibit increased weight gain than wildtype when fed a normal mouse diet. Mammary glands were dissected from *Alms1bbb/bbb* and wildtype female mice during puberty (6 weeks; n=10/gp). To determine the impact of pubertal adiposity on mammary density, *Alms1bbb/bbb* mice were calorie-matched with wildtype from 7 weeks of age, such that weight of adult *Alms1bbb/bbb* mice was comparable to that of wildtype. Mammary glands were then dissected from calorie-

restricted *Alms1bbb/bbb* (and controls) at adulthood (12 weeks; n=10/gp). *Alms* mice were crossed with *Mmtv-PyMT* tumour mouse model (18 weeks; n=15/gp) to determine the impact of pubertal adiposity on mammary tumour development.

At puberty, *Alms1bbb/bbb* mice exhibited increased number of terminal end buds and proliferating epithelial cells compared to wildtypes, as well as larger adipocytes and increased number of macrophages around terminal end buds and in the mammary adipose tissue. At adulthood, *Alms1bbb/bbb* mice exhibited a 56% decrease in fibroglandular density, accompanied with reduced stroma and collagen deposition, compared to wildtype. A 46% decrease in tumour burden and delayed tumour development was observed in *Alms1bbb/bbb-PyMT* mice, compared to control-*PyMT* mice.

Our findings indicate that increased adiposity during puberty reduces both mammary fibroglandular density and cancer development in adulthood. Together with epidemiological studies, this research provides the foundation for a new paradigm for the origins of mammographic density and breast cancer risk during pubertal mammary gland development.

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Telomere elongation during embryogenesis is regulated by oocyte mitochondria

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Telomeres are protective DNA sequences at chromosome ends that control cellular proliferation potential and senescence. Telomere length is an important determinant of health, with short telomeres associated with diminished tissue function and short lifespan. Telomeres shorten with every cell division, therefore telomere length must be regenerated in offspring to ensure viability of each new generation, however exceedingly little is known about this process and how it is regulated. Characterisation of telomere length in individual mouse oocytes and embryos using a novel qPCR assay revealed elongation occurs rapidly within the first three cell divisions and again at the blastocyst stage. Parthenotes exhibited telomere elongation indicating the necessary factors are present in oocytes. We tested whether oocyte mitochondria regulated this process by measuring telomere elongation in mice with compromised mitochondrial activity and in response to mitochondrial activators. Aged or obese female mice exhibit reduced oocyte mitochondria membrane potential (using TMRM potentiometric dye), that is similarly reduced by *in vivo* rotenone (Complex I inhibitor) exposure or high oxygen embryo culture. Telomere length per cell was reduced in blastocysts, specifically in the inner cell mass (ICM), from each of the mitochondria dysfunction models compared to controls, indicating deficient telomere resetting in these embryos. Following embryo transfer, shorter telomeres were maintained in fetal tissues, predicting shorter lifespan in these offspring. Aged or obese female mice were treated with compounds known to activate oocyte mitochondria (BGP-15, Metformin, MitoQ) prior to ovulation to determine if the deficiency is reversible. Remarkably, mitochondria-activating compounds prior to fertilisation restored telomere lengths in ICMs of aged and obese females to similar levels as those from young lean controls. Thus, embryo telomere resetting is impaired in the presence of mitochondria dysfunction, including with female obesity and reproductive ageing, and likely contributes to poorer health outcomes, including reduced lifespan, documented in these offspring.

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Interleukin-1 is overexpressed in injured muscles following spinal cord injury and promotes neurogenic heterotopic ossification

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Neurogenic heterotopic ossifications (NHOs) are pathological heterotopic bones developing in periarticular muscles following central nervous system injuries including spinal cord injuries (SCI) and traumatic brain injuries (TBI). The pathobiology of NHO is poorly understood, hence the curative treatment is limited to surgical resection of pathological NHOs. Using a SCI-induced NHO mouse model with dual insults combining a spinal cord transection and a muscle injury via intramuscular injection of cardiotoxin (CDTX), we have demonstrated that macrophages-mediated inflammatory responses are required for NHO formation. In this study, we investigated the changes in muscle microenvironment that may promote NHO formation using microarray gene expression analyses on whole muscle mRNA extracts. Gene set enrichment analysis (GSEA) showed that the inflammation gene set was significantly enriched in muscles from mice with SCI and CDTX-mediated muscle injury developing NHO compared to muscle injury alone, SCI alone, or sham-SCI control groups that do not develop NHO. Genes encoding inflammatory cytokines such as interleukin-1β (IL-1β) were overexpressed in muscles developing NHO compared to muscles injured with CDTX alone. NHO development was reduced in mice with defective *Il1r1* gene encoding IL-1 receptor. This suggests IL-1 signaling contributes to NHO development following SCI in mice. Interestingly, some other genes involved in inflammation, such as colony-stimulating factor-1 (CSF1), tumor necrosis factor (TNF) or C-C chemokine ligand-2 (CCL2), were also upregulated in muscles developing

NHO; however, they were not critical for NHO development. Of relevance to the human pathology, IL-1β was expressed by $CD68^+$ macrophages and osteoclasts in NHO biopsies from SCI and TBI patients. In vitro study demonstrated that IL-1α and IL-1β produced by activated human monocytes promoted calcium mineralization of fibro-adipogenic progenitors isolated from muscles surrounding NHOs. In summary, these data suggest that interleukin-1 signalling contributes to NHO development in both humans and mice.

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Leveraging osteoclast genetic regulatory mechanisms to identify genes with a role in osteoarthritis

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Osteoarthritis (OA) is the most prevalent form of arthritis and is characterised by deterioration of the articular cartilage within a joint. Abnormal remodelling of the subchondral bone underlying this cartilage is thought to have a major role in the disease. OA has a significant genetic component, with twin studies suggesting the heritability of radiographic knee and hip OA to be around 39% and 60% respectively. Genome-wide association studies (GWAS) have identified at least 64 genetic loci associated with the disease.

We have generated a unique expression quantitative trait locus (eQTL) resource for mapping genetic regulatory regions in osteoclasts using cells differentiated *in vitro* from 158 patients undergoing bone mineral density scanning at Sir Charles Gairdner Hospital in Western Australia. Considering the role of subchondral bone remodelling in OA, we used this resource to investigate the 64 OA GWAS loci for evidence of genetic regulatory effects relevant to osteoclasts.

After correction for multiple testing, in the osteoclast dataset we observed significant associations between the OA GWAS variants rs11732213 (4p16.3), rs2953013 (17q11.2) and rs143384 (20q11.22) and expression of the genes *FAM53A*, *OMG* and *UQCC1* respectively ($P=5.6\times10^{-5}$– 1.4×10^{-6}). Strong evidence for co-localisation (>75% posterior probability) of OA GWAS and osteoclast genetic regulatory association signals was observed for all 3 loci. In each instance, the OA GWAS variant was in close proximity (within 150kb) to the eQTL-gene transcription start site. *UQCC1* presents strongly as an OA-risk gene, encoding a growth regulator and having previously been implicated in developmental dysplasia of the hip.

We have identified regulatory effects for the OA GWAS variants rs11732213 (*FAM53A*), rs2953013 (*OMG*) and rs143384 (*UQCC1*) in human osteoclasts. This study highlights the value of using genetic regulatory data to determine which genes are relevant to GWAS loci.

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Molecular Transporters in Bone Homeostasis and Disease

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The transport of molecules across biological membranes is a vital process for all aspects of cellular function and tissue homeostasis. Membrane transporters are the molecular gates that control this transport and serve as key points of cellular regulation, thus representing an attractive class of therapeutic targets. Mutations in these transporter systems have been increasingly implicated in a wide variety of metabolic diseases, including those that extend to bone. Bone resident cells express their own unique complement of transporters that are enriched on the cell surface as well as in membranes of intracellular organelles. This is best exemplified in osteoclasts whose specialised secretory organelles are equipped with structurally and functionally diverse membrane transport nanomachinery uniquely adapted to digest mineralised bone. Despite their obvious importance, our understanding of membrane transporter systems in bone homeostasis and disease remains in its infancy. In particular, compared to other mammalian systems, the nature and number of transporters that reside and operate on bone cell membranes remains poorly understood. This presentation will provide a brief overview of our current understanding of molecular transport systems in bone homeostasis and disease, with a focus on membrane transporters in osteoclasts. It will unveil new and unexpected transporters in osteoclasts and highlight their potential as therapeutic drug targets for the treatment of metabolic bone diseases.

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Mimicking the Dragonfly Wing-Like Structure as an Antimicrobial Surface for Orthopaedic Devices

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Periprosthetic Joint Infection (PJI) is defined as an infection involving the joint prosthesis and adjacent tissue. More than 1% of primary hip and 1 to 3% of primary knee arthroplasties fail due to PJI leading to ongoing morbidities and mortality. A recent discovery that the dragonfly wing surface structure naturally kills bacteria has led to recreating this passive nano-textured surface on titanium alloy (Ti6Al4V). The aim of this study was to assess the anti-microbial and osseointegration properties of the nano-textured Ti6Al4V implants using *in vitro* and pre-clinical *in vivo* models. Nano-textured Ti6Al4V, when incubated with *Staphylococcus aureus* (*S. aureus*) or *Pseudomonas aeruginosa* (*P. aeruginosa*), exhibited 60% and 98% reduction in live cells respectively, when compared to levels on control Ti6Al4V surface. To validate these findings, 12-week-old male rats underwent bilateral hindlimb surgery to insert rod-shaped implants (nano-textured or control Ti6Al4V) pre-coated with *S. aureus* (10^2-10^4 colony forming units (CFUs)) into each femur. Secondly, 8-week-old mice, received disc or pill-shaped implants pre-coated with 10^4 CFUs *S. aureus* and inserted in the subcutaneous space, to assess activity in a soft tissue environment. Implants from these two *in vivo* models are under evaluation for bacterial load, confocal and SEM imaging. Lastly, to assess osseointegration, the nano-textured Ti6Al4V implants were surgically inserted into tibial cortical and femoral cancellous bone in sheep, with implant integration assessed after 12-weeks using histomorphometric and biomechanical push-out analyses. When compared to porous ceramic hydroxyapatite (PCHA)-coated commercially available control implants, nano-textured Ti6Al4V implants had a significantly higher cortical bone contact surface, interface shear strength, peak load, failure load, and proof resilience, when compared to the PCHA coated implants. To date, these studies suggest that the nano-textured Ti6Al4V surface has clinical potential for medical devices that have orthopaedic applications and anti-microbial properties.

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Motion is essential to maintain the synovial condition and joint homeostasis

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Motion has a crucial role in joints. Long-term joint immobilization causes contracture and cartilage degeneration, whereas joint motion exercise is effective to alleviate osteoarthritis symptoms. However, the molecular mechanisms of these effects have been unclear. Here, we investigated how joint motion regulates joint homeostasis. We first established a minimized mechanical stress (MMS) model by knee joint immobilization of mice that were suspended by their tails. Histological examination showed synovitis reached a peak at 2 weeks, while cartilage degeneration had occurred by 6 weeks. When we cancelled immobilization at 2 weeks, synovitis was reversed and cartilage degeneration was prevented. Bulk RNA-seq of the synovium and cartilage of MMS model mice showed remarkable increases in expression of catabolic factors in synovium and remarkable decreases of matrix proteins in cartilage. On the basis of the cartilage gene expression alterations, Ingenuity Pathway Analysis estimated dozens of upstream cytokines and growth factors, including *Spp1* and *Il-1β*, whose expressions were increased in the MMS model synovium. We also conducted scRNA-seq of synovium from control and MMS model mice. A few specific subsets were identified among activated fibroblasts and activated macrophages, which remarkably increased in the MMS model synovium. These subsets expressed cytokines and growth factors responsible for cartilage degeneration, which was consistent with the bulk RNA-seq and histological findings. Moreover, ligand-receptor analysis indicated dynamic interactions between the two MMS-specific subsets through these secreted molecules. In summary, loss of mechanical loading on joints induces specific subsets of activated fibroblasts and macrophages in synovium, which probably cause cartilage degeneration through secretion of catabolic and inflammatory factors. The synovial changes are reversible by resuming joint motion, which indicates that the two MMS-specific subsets or the pathological interaction between them are mechanosensitive. Taken together, we conclude that motion is essential to maintain the synovium and consequently joint homeostasis.

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The Protective Role of ACE2 in Pregnancy

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Intrauterine growth restriction (IUGR) and preeclampsia (PE) are common and life-threatening complications for mothers and babies. Currently there are no effective treatments other than delivery of the baby. We postulate that angiotensin converting enzyme 2 (ACE2) is important in protecting against placental inflammation and oxidative stress, preventing PE and IUGR.

We have recently shown that ACE2 is abundant in the healthy human placenta and soluble ACE2 levels are elevated in pregnancy but its expression is reduced in pregnancies complicated by IUGR and in the circulation of women with PE. ACE2 is well known to have protective roles in counterbalancing the vasoconstrictor, pro-inflammatory and oxidative stress inducing arm of the renin-angiotensin system (RAS), which is mediated by angiotensin II (Ang II), however its protective actions in pregnancy have not yet been explored.

Reduced placental production of ACE2 as seen in IUGR and, for example, as a result of nutritional deficiency, stress or COVID-19 infection, is associated with placental inflammation and oxidative stress and thus may contribute to the pathogenesis of IUGR and PE. ACE2 is the receptor for the novel coronavirus, SARS-CoV-2, which causes COVID-19. Since SARS-CoV-2 decreases placental expression of ACE2, further investigation into the consequences of COVID-19 infection in the mother and her fetus are needed. Not only do women with COVID-19 infection during pregnancy have worse respiratory outcomes but they are also more likely to develop gestational hypertension and PE. Furthermore, the risk of low birth weight and intrauterine fetal distress is increased.

We propose that loss of the protective actions of ACE2 causes placental inflammation, oxidative stress, fetal growth restriction and maternal hypertension. Therefore, recombinant ACE2 or activation of the ACE2-mediated RAS pathways could be potential treatments for PE and IUGR and for preventing PE and IUGR in pregnant women with COVID-19.

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Respiratory Viral Infections and Diabetes

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The COVID-19 pandemic has highlighted the vulnerability of people with diabetes mellitus (DM) to respiratory viral infections. Despite the short history of COVID-19, various studies have shown that patients with DM are more likely to have increased hospitalization and mortality rates as compared to patients without. At present, the mechanisms underlying this susceptibility are unclear. . The reasons for this are likely multifactorial, including the presence of hyperglycaemia, glycaemic variability as well as selected medication use. Here, I present our most recent data showing the mechanisms by which type 2 diabetes mellitus impairs anti-viral immunity and increases disease severity.

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The Intersection of AR Signalling and COVID-19

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A cluster of unexplained pneumonia in late-2019 was the preface to a rapid-spreading global health crisis sparked by emergence of a novel coronavirus (SARS-CoV-2). Since its emergence, the highly contagious SARS-CoV-2 illness Coronavirus disease 2019 (COVID-19) has affected millions of people worldwide and triggered a rapid scientific movement to map case statistics, understand disease pathophysiology and find efficacious treatments for this insidious disease. Clinical case studies consistently report that being male is a major risk factor for COVID-19 morbidity and mortality. In one study covering 1099 adults admitted to 552 mainland China hospitals, 70% of patients requiring ventilation support in intensive care were biologically male (Guan et al, 2020, N Eng J Med). This was corroborated in reports of COVID-19 mortality across 38 countries, where the fatality rate of men was 1.7-fold higher than women (Scully et al, 2020, Nat Rev Immunol). Albeit, in paediatric studies this gender gap was not evident; prepubescent females and males were equally impacted by mild/moderate COVID-19 (Wu et al, 2020, JAMA Netw Open). These observations of distinct sex-specific disparities in the course of COVID-19 disease have triggered rigorous inquiry into the role of sex hormones in driving SARS-CoV-2 virus susceptibility and illness severity. Evolving evidence suggests male sex hormones, androgens, regulate the SARS-CoV-2 receptor, Angiotensin-Converting Enzyme 2 (ACE2), and co-receptor, Type II Transmembrane Serine Protease (TMPRSS2), which facilitate SARS-CoV-2 entry and infection of host cells. Furthermore, the host's sex hormone milieu influences the immune response mounted in response to SARS-CoV-2 respiratory infection and the release inflammatory cytokines including interleukin-6 (IL-6) which is markedly elevated in severe COVID-19 infection and exhibits excessive secretion in males. In conclusion, the emerging intersection between androgens and COVID-19 may pave the way for hormone rationalised therapies to effectively lower disease severity across patients affected by severe COVID-19 illness.

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ACE2 and Vascular Health Including Recent Studies on COVID-19

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Available Soon

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Determining Embryo Health with a Light Touch

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Euploid/aneuploid mosaicism affects up to 17.3 % of human blastocyst embryos with trophectoderm biopsy or spent media currently utilised to diagnose aneuploidy and mosaicism in clinical *in vitro* fertilisation. Based on their design, these approaches will fail to diagnose the presence of aneuploid cells within the fetal lineage (inner cell mass (ICM)) of some blastocyst embryos. In this study we determined whether non-invasive hyperspectral autofluorescence microscopy can discern between euploid and aneuploid cells within the ICM of mouse preimplantation embryos.

Mouse embryos were treated with reversine, a reversible spindle assembly checkpoint inhibitor, during the 4- to 8-cell division. Individual blastomeres were dissociated from control and reversine-treated 8-cell embryos and imaged directly or used to generate chimeric blastocysts with differing ratios of control: reversine-treated cells. Individual blastomeres and embryos were interrogated by hyperspectral imaging. Changes in metabolism were determined by quantification of metabolic co-factors: reduced nicotinamide adenine dinucleotide (NAD(P)H) and flavins, with subsequent calculation of optical redox ratio (ORR:

Flavins/[NAD(P)H + Flavins]). Separately, autofluorescence signals obtained from hyperspectral imaging were examined mathematically to extract features. This was used to discriminate between different cell populations.

An increase in the abundance of NAD(P)H and decrease in flavins led to a significant reduction in the ORR for reversine-treated mouse blastomeres ($P < 0.05$). Mathematical analysis of cell autofluorescence achieved separation between (i) control and reversine-treated mouse blastomeres cells, (ii) control and reversine-treated chimeric blastocysts, (iii) 1:1 and 1:3 chimeric blastocysts and (iv) confirmed euploid and aneuploid ICM from mouse blastocysts. The accuracy of these separations was supported by receiver operating characteristic curves with areas under the curve of 0.99, 0.87, 0.88 and 0.93, respectively.

Hyperspectral autofluorescence imaging was able to discriminate between euploid and aneuploid ICM in mouse blastocysts. This approach may lead to an accurate and non-invasive diagnostic for embryo analysis.

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Maintenance and regeneration of the male germline

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Maintenance of male fertility is dependent on spermatogonial stem cells (SSCs) that self-renew and generate differentiating germ cells for production of spermatozoa. SSC function is dependent on growth factors produced within the testis microenvironment plus cellular factors that regulate gene expression within SSCs and modulate responses to growth factor stimulation. Despite the importance of SSCs for male fertility, the molecular mechanisms that regulate their function and maintenance remain incompletely understood. Importantly, SSC function and male fertility can be compromised by multiple factors including exposure to genotoxic drugs. However, cellular pathways mediating the regenerative response of SSCs following germline damage and loss of SSC function with age are poorly studied. Our research focuses on defining genetic controls and cellular pathways regulating SSC function and male fertility. We employ a range of in vivo and in vitro experimental systems allowing dissection of mammalian SSC function. We have defined essential roles for the developmental transcription factors PLZF and SALL4 in maintenance of SSC activity and the central importance of mTORC1 signalling in SSC fate regulation. In addition, our studies have characterised cellular heterogeneity within the SSC and progenitor cell pool using single cell approaches and demonstrated the dynamic nature of spermatogonial states with important clinical implications. Current studies are focused on understanding cellular machinery modulating the response of SSCs to stimuli from the niche and molecular mechanisms supporting germline regenerative capacity.

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Medical Microrobots: A New Paradigm Shift Towards In-Vivo Assisted Fertilization

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Novel concepts of untethered microrobots have been developed worldwide to realize non-invasive medical tasks in biological-relevant scenarios. Potential geometries of such tiny robots range from microcages, spheres, to bio-inspired artificial flagella and are designed to transport drugs, cells or molecular reporters to realize targeted therapies. With a similar scope, we have developed different types of biohybrid and bioinspired micromotors, in particular, sperm-hybrid microrobots and microcarriers for fertilized oocytes with the purpose of increasing the pregancy success rate and to reduce the invasiveness of current assisted fertilization technologies. We have successfully demonstrated the guidance and transport of motile and immotile sperm by magnetic microcarriers actuated by weak external magnetic fields, in vitro, employing biological-relevant fluids. These sperm-hybrid microrobots have also been suggested for the first time as potential drug carriers towards gynecological cancer treatment, in which different species of sperm cells from mouse, bull and even human, have been succesfully loaded with anticancer drugs to realize sub-cellular drug delivery. Moreover, we succeeded in the transport and release of multiple viable and mature sperm, employing different carrying strategies, being a crucial step to achieve the egg fertilization in vivo or to control drug dose in the case of cancer therapy. We have also evaluated their perfomance under blood stream as sperm have the ability to swim against flow, and exploited their cargo-delivery ability by functionalizing the carriers with heparin-loaded nanoliposomes. Finally, in order to translate these technologies to pre-clinical trials, we have recently reported the succesful tracking of magnetically-driven micromotors in phantom, ex-vivo and in living mice with high spatial and temporal resolution employing photoacoustic and high frequency ultrasound imaging.

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Novel Technologies in Reproduction

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Eudaemon Technologies is a start-up building next generation sexual and reproductive health innovations utilising novel material science, user driven design, and advanced manufacturing. Our first product innovation, ‘Project Geldom’, is bringing Industry 4.0 to the condom industry, a growing $8B business producing 27B condoms annually with the potential to improve SRH for billions of users and alleviate the estimated $60 global burden from ineffective condom use. Eudaemon is replacing legacy latex with materials like tough hydrogels to create better feeling, safer and more-cost effective condoms to revolutionise safe sex, reduce STIs and promote family planning globally. With our custom compound-delivery material platform and industrial product development process established, Eudaemon is now primed for expansion into assisting the reproductive health arena. This talk will explore the relationship in development between contraception and reproduction and potential opportunities for work in Australia.

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Identifying monogenic causes of male infertility

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Globally, 7% of men are diagnosed with infertility, and for approximately half of these men a causal diagnosis is unknown. We hypothesise that at least 50% of these have a genetic element of causation, and that these genetic causes will be primarily comprised of single gene mutations. To identify such monogenic causes, we performed whole exome sequencing on 185 men diagnosed with azoospermia or severe oligozoospermia and their parents. This trio-based analysis allowed us to investigate both homozygous and compound heterozygous causes of disease.

Sequencing data was aligned to the reference genome and variants were annotated with their predicted effect on protein product, allele frequency in gnomAD and their predicted pathogenicity. Variant were prioritised based on their expression in the testis, published functional data, whether they interacted with known fertility genes, and whether orthologs were known to cause a mouse fertility phenotype.

We identified 62 predicted pathogenic recessively inherited bi-allelic variants, affecting 62 protein-encoding genes. Six of these bi-allelic variants were in genes already known to cause human male infertility; however, the clinical presentation was different from reported previously for 5 of the patients affected. The other 56 bi-allelic variants affected genes not previously associated with human male infertility. To explore functional links between the identified genes, we used the STRING database to illustrate protein-protein interactions between candidate infertility genes and found that genes involved in DNA repair and piRNA biogenesis were enriched within our cohort.

Together these results add evidence to the emerging role for monogenic mutations in male infertility. Providing patients with a molecular diagnosis will allow for a greater clarity of genetic counselling, including expected consequences of ART, for both reproductive outcomes and offspring health. This research is expected to lead to a shift in clinical practice towards genome sequencing for diagnostics.

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AXDND1 is a novel dynein adapter protein essential for sperm production and function

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Male infertility is a common condition affecting at least 7% of men worldwide and is often genetic in origin. Using whole exome sequencing, we have identified a high confidence infertility-causing mutation in *AXDND1* in a man with azoospermia. This 'stopgain' mutation affects the putative axonemal dynein light chain domain and is predicted to cause a complete loss of AXDND1 function. *Axdnd1* is highly testis enriched in mice and men, and in male germs is largely expressed in spermatocytes and spermatids. We generated *Axdnd1* knockout mice with a premature stop codon in exon 3 to further explore the role of AXDND1 in male fertility. *Axdnd1* knockout mice were infertile and presented with a multifaceted phenotype that worsened with age. At 7 weeks of age, just after the first wave of spermatogenesis and epididymal maturation, spermatogenesis was intact. Normal numbers of sperm were present in the cauda epididymides of knockout males, but all were completely immotile. Electron microscopy revealed the axonemes of sperm from knockout males to be severely disrupted, with key accessory structures (outer dense fibres, microtubule doublets) missing. By 10 weeks of age there was a significant loss of germ cells in 15% of tubules, a complete loss of germ cells in 5% of tubules, and an increase in the immune cell population in the intertubular space of *Axdnd1* knockout testes. This translated to a 99.3% reduction in epididymal sperm count compared to wildtype, and the presence of precociously sloughed germ cells and immune cells in the cauda epididymis. Although predicted to be an axonemal dynein protein based on the possession of an axonemal dynein light chain domain, our data suggest AXDND1 primarily plays roles in cytoplasmic dynein function in male germ cells. Specifically, we hypothesise AXDND1 is required for cargo transport during spermatogenesis, including into the developing sperm tail.

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Investigation into the sperm proteomic profiles of fertile, infertile, and severely sub-fertile stallions reveals several putative biomarkers of fertility

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When a retired racing stallion is selected for breeding, their reproductive fitness is generally unknown. Where a severely sub-fertile or infertile stallion commences an ill-fated breeding career, it can generate a host of unnecessary economic and welfare costs, stemming from futile, repeated breedings. To date there are no effectual processes in place to tackle this problem. As such, this study aimed to compare the proteomic profiles of spermatozoa collected from stallions of variable fertility status, to identify robust biomarkers of fertility that could be used as pre-purchase diagnostic tools, thereby identifying unsuitable sires in the future.

Semen samples were collected from two infertile, four sub-fertile (conception rates 0–33%) and 15 commercially 'fertile' stallions (conception rates 61.5–100%), and assessed using LC-MS/MS. This analysis identified a complex, proteomic signature comprising 1,076 proteins; of which 37 proteins were more abundant in samples collected from sub-fertile and infertile stallions. These included α-aminoadipic semialdehyde dehydrogenase (ALDH7A1) and voltage-dependent anion-selective channel protein 3 (VDAC3;FC≥-1.5;P≤0.0001). When comparing the proteomic conservation between the fertile and infertile stallion ejaculates, a notable 79 proteins were absent from infertile samples. These included isocitrate dehydrogenase 3 (NAD+)-α (IDH3A); stress-70 protein, mitochondrial (HSPA9); arylsulfatase A (ARSA); and phospholipase C zeta 1 (PLCZ1), which are individually associated with asthenospermia, fertilisation and oocyte activation. Conversely, a comparison of fertile and severely sub-fertile stallion ejaculates revealed 113 proteins were exclusively conserved to fertile ejaculates. These included testisin (PRSS21); α-enolase (ENO1); apolipoprotein E (APOA1); and tektin-3 (TEKT3), which are associated with zona-pellucida binding, fertility, and varicocele.

We have identified a suite of putative protein biomarkers that have substantial potential for assessing stallion reproductive fitness. These findings will serve to inform the development of novel diagnostics to improve pregnancy success rates, thereby dramatically reducing the economic wastage and welfare implications associated with futile breeding practices.

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Elevated paternal glucocorticoids preconception contributes to intergenerational shifts in male attractiveness and major urinary protein expression

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Previous studies from our lab modelling chronic stress exposure through paternal corticosterone supplementation preconception revealed altered anxiety and depression-relevant behaviours in male progeny. Given the strong presence of sociability deficits in various affective disorders, we sought to characterise social behaviour across generations in our model. Additionally, we had reported significant changes to sperm sncRNA content associated with corticosterone supplementation but DNA methylation, a key epigenetic modification, had yet to be investigated.

Two generations of adult male progeny derived from C57BL/6J male mice treated with corticosterone for 4 weeks prior to paired-matings were assessed using the Mate-Choice Test. This involved a modified 3-chamber interaction test, where female mice on oestrous explored the apparatus while the males were contained, indicating their relative attractiveness. Protein concentration and gene expression of the male pheromone Major Urinary Protein (MUP) were quantified in urine and liver, respectively. Sperm from corticosterone-treated mice were harvested from the caudal epididymis and DNA was extracted, then processed for Oxford Nanopore long-read sequencing, followed by in-house bioinformatic analyses to detect differential methylation.

Paternal corticosterone exposure was associated with increased female attraction towards male offspring (PatCORT), with no differences observed for grand offspring (GPatCORT). These observations were not attributable solely to an overall change in urinary MUP protein levels. However, specific MUP subtypes (MUP20 and Major MUP bands) were found to be decreased in PatCORT urine. No differences in these MUP subtypes were found from GPatCORT urine. Interestingly, we found that corticosterone-treatment resulted in altered sperm DNA methylation in regions proximal to Mup genes.

Paternal stress preconception may influence social behaviour intergenerationally, as we found altered male attractiveness across one generation of progeny. These changes were unexpectedly accompanied by lower urinary MUP levels in the male offspring. Further investigations into the male PatCORT response to female urine could clarify these results.

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KATNB1 is a master microtubule regulator in male meiosis and haploid germ cell development

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Katanin microtubule-severing enzymes are key microtubule regulators. Previously, we showed a hypomorphic mutation in the katanin regulatory B-subunit *Katnb1* disrupts germ cell microtubule dynamics resulting in production of abnormal sperm. Herein, we sought to define the full range of KATNB1 spermatogenesis functions using a graded series of KATNB1 loss-of-function (LOF) mouse models. This consisted of *Katnb1* hypomorphic (*Katnb1*$^{Taily/Taily}$), compound heterozygous *Katnb1*$^{Taily/KO}$, and *Katnb1* germ cell specific KO (*Katnb1*$^{GCKO/GCKO}$) mice.

Spermatogenesis was abnormal in all KATNB1 LOF models. In the new *Katnb1*$^{Taily/KO}$ and *Katnb1*$^{GCKO/GCKO}$ models however, more severe and additional phenotypes emerged. Reductions in spermatogenic output scaled with KATNB1 expression, with complete KATNB1 LOF being incompatible with germ cell survival and sperm production (59.9% reduction in *Katnb1*$^{Taily/Taily}$ daily sperm output, 83.6% in *Katnb1*$^{Taily/KO}$, and 91.9% in *Katnb1*$^{GCKO/GCKO}$). The reduced *Katnb1*$^{Taily/Taily}$ spermatogenic output was due to spermatocytes stalling in anaphase and cytokinesis, resulting in fewer spermatids. In *Katnb1*$^{Taily/KO}$ and *Katnb1*$^{GCKO/GCKO}$ however

the points of germ cell loss were multi-fold and included loss due to catastrophic defects in meiosis and spermatid remodelling, and premature germ cell sloughing from the disordered seminiferous epithelium. Notably, *Katnb1*$^{Taily/KO}$ and *Katnb1*$^{GCKO/GCKO}$ meiosis frequently failed in metaphase, as well is in anaphase and cytokinesis, due to defects in spindle architecture and dynamics. During haploid germ cell development, more severe loss of KATNB1 function resulted in defects in acrosome biogenesis principally due to abnormal vesicle trafficking. Concomitantly, ectopic vesicles accumulated within the sperm tails of all KATNB1 LOF models, suggestive of intraflagellar transport defects. All KATNB1 LOF models also exhibited abnormal sperm head shaping due to dysregulation of the manchette and, in *Katnb1*$^{Taily/KO}$, the Sertoli cell cytoskeleton. Collectively, this study establishes KATNB1 as a master microtubule-severing regulator during spermatogenesis, required for the regulation of almost all microtubule-based structures.

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Constitutive deletion of NFIX results in defective progression through meiosis within the mouse testis

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Members of the Nuclear Factor I family (NFI) are key regulators of stem cell biology during development, with well documented roles for NFIA, NFIB and NFIX in a variety of developing tissues, including brain, muscle and lung. Given the central role these factors play in mediating stem cell biology in various systems, we posited that they might also be pivotal for spermatogonial stem cells during testicular development. Surprisingly, in stark contrast to other developing organ systems where NFI members are co-expressed, we revealed that three NFI family members show discrete patterns of expression within the seminiferous tubules. Sertoli cells (spermatogenic supporting cells) express NFIA, spermatocytes express NFIX, round spermatids express NFIB, and peritubular myoid cells express each of these three family members. Further analysis of NFIX expression during the cycle of the seminiferous epithelium revealed expression not in spermatogonial stem cells, as we anticipated, but in pre-meiotic spermatocytes. These data suggested a potential role for NFIX in spermatogenesis so we investigated mice with constitutive deletion of *Nfix* (*Nfix-/-*). Assessment of germ cells in the postnatal day 20 (P20) testes of *Nfix*$^{-/-}$ mice (*Nfix-/-* mice do not survive past P22) revealed that spermatocytes initiate meiosis, but zygotene stage spermatocytes display structural defects in the synaptonemal complex, and increased instances of unrepaired DNA double-strand breaks. We found that many developing spermatocytes exhibited multinucleation, cytokinetic defects, as well as a significant increase in the number of apoptotic cells in the *Nfix*$^{-/-}$ testes, compared to controls. As a result of these defects, spermatogenesis arrests at early diplotene and very few round spermatids were observed. Collectively, these novel data establish the global requirement for NFIX in correct meiotic progression during the first wave of spermatogenesis.

ESA POSTER ABSTRACTS

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A combination therapeutic approach to GCT

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Granulosa cell tumours (GCT) are uncommon ovarian cancers that are characterised by an indolent clinical course and significant rates of late recurrence. Furthermore, these tumours are unique from other ovarian cancers with a majority secreting estrogen and inhibin. Aside from invasive surgery, there are limited therapeutic options. Chemotherapy is not effective and associated with severe adverse effects, highlighting the need for targeted therapies in GCT.

Our laboratory has previously shown that the X-linked Inhibitor of Apoptosis protein (XIAP) inhibitors, Smac-mimetics, are an effective combination agent in GCT. XIAP inhibition sensitises cancer cells to anti-cancer therapies through regulating key pro-survival pathways, namely NFkB.

We hypothesised that XIAP inhibition using Smac-mimetics (SM) combined with established drugs targeting additional pathogenic pathways will provide a novel therapeutic strategy for GCT. To determine the most effective combination agents, we performed a high-throughput drug screen (HTS) using both an FDA-approved and an anti-cancer compound library (1μM each compound) +/- SM (500nM). We used two cell lines, KGN (GCT-derived) and hGrC1 (transformed normal granulosa cells). Cell viability was determined using an alamarBlue assay following 72 hours of treatment. We selected two compounds, YM155 (survivin inhibitor), and Panobinostat (HDAC inhibitor) for further investigation. We demonstrated using cell proliferation and viability assays, that YM155 is highly effective as a single agent at 50nM. Additionally, 100nM Panobinostat acted synergistically with 500nM SM. The use of both agents led to an increase in apoptosis at these same concentrations, as demonstrated by increased caspase 3/7 activity. We are currently validating these results by assessing apoptosis using flow cytometry. Further aims will be to determine the mechanism of action of these compounds by investigating the expression of key components of the NFkB signalling pathway. These results represent two different promising therapeutic strategies for GCT, leading to potential clinical translation.

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Dietary fats and the implication of type 2 diabetes

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Consumption of a western style high saturated fat diet (HSFD) has been implicated in obesity development as well as progression to Type 2 diabetes. Chronic consumption of HSFD exerts detrimental effects on pancreatic islet function in mice. Whether monounsaturated and polyunsaturated fat are less detrimental for human islet function is also unknown.

AIM: To investigate the impact of different forms of high fat diet on mouse pancreatic islet functions.

METHOD:

Male RAG1-null mice (C57Bl/6 background) at age of 6-8 weeks were placed on one of 4 different diets: normal chow, high saturated fat diet (HSFD), high monounsaturated fat diet (MUFD; fat ratio 3:2:2 being MUFA: PUFD: SFA), or high polyunsaturated fat diet (PUFD; omega 3 to omega 6 fatty acid being 1:3.7). All high fat diets have 45% calories from fat, chow has 8% of calories from fat; n=10 on each diet.

Glucose tolerance tests and insulin tolerance tests were performed before and 16 weeks after diet change. Mice were also placed in a promethion metabolic cage post diet.

RESULTS:

HSFD fed mice had substantial weight gain ($p<0.0001$ vs chow) along with significant glucose intolerance and insulin intolerance ($p<0.005$) by 16-week. In contrast, mice fed MUFD or PUFD had insignificant and similar weight gain to mice fed chow ($p=0.5852$ chow vs MUFD; $p=0.9998$ for chow vs PUFD). On MUFD and PUFD, glucose tolerance did worsen compared to chow, but with much smaller effect compare to HSFD. Higher energy expenditure was found in mice fed MUFA / PUFD while their food consumption was similar across diets.

CONCLUSION:

Long-term unsaturated-high-fat diet demonstrated significant improvements in body weight and islet function in comparison to HSFD. This suggests replacing saturated fats with unsaturated fats could potentially lower the risks of type 2 diabetes development.

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The development of total inhibin assay for monitoring ovarian cancer recurrence using mass spectrometry

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Inhibins are gonadal glycoprotein hormones belonging to the transforming growth factor-beta superfamily. They are dimers composed of a common α-subunit and either a βA or a βB subunit, forming either bioactive inhibin A or inhibin B respectively. They play a major role in the hypothalamus-pituitary-gonad axis regulating spermatogenesis and folliculogenesis through negative feedback on FSH secretion. Total inhibin (inclusive of all bioactive forms and free α subunits) is an excellent marker for granulosa cell tumours (GCTs) which are the most common type of ovarian sex cord stromal cancer. This is especially true for postmenopausal women with GCT as inhibin levels are generally undetectable in healthy postmenopausal women. GCT have a tendency for late recurrence, often many years after initial diagnosis; inhibin is valuable for monitoring for ovarian cancer recurrence. An efficient and cost-effective diagnostic assay for total inhibin is not available, underscoring a need to develop a high-throughput total inhibin assay. Mass spectrometry in clinical endocrinology is playing an increasing role in improving the clinical management of numerous endocrine diseases. Our aim is to establish a methodology using selective reaction monitoring (SRM)-based targeted proteomics using liquid chromatography-mass spectrometry (LC-MS) to detect total inhibin with enhanced sensitivity in serum. Using conditioned media from HEK293 cells that overexpress inhibin B, we successfully detected inhibin α peptides using a timsTOF Pro mass spectrometer. STPLMSWPWSPSALR was the highest-scoring detectable peptide sequence at 74.26 (2142.1156 second retention time). Using these parameters, in conjunction with an immunoaffinity enrichment method, we are currently testing serum samples collected from women who have had GCT and comparing them to a standard inhibin α ELISA assay. This study will provide women with GCT access to an inhibin assay with high sensitivity and specificity with the aim to achieve early detection or monitoring of recurrence and an improved overall survival rate.

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A high-throughput imaging assay for drug screening of 3D prostate cancer organoids

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Background: New treatments are required for advanced prostate cancer; however, there are fewer preclinical models of prostate cancer than other tumour types to test candidate therapeutics. One opportunity to increase the scope of preclinical studies is to grow tissue from patient-derived xenografts (PDXs), human cancer samples grown in mice, as organoids - 3D *in vitro* cultures embedded in Matrigel. Indeed, prostate cancer organoids have been used to test drug libraries or specific therapeutics. However, these assays provide only limited, endpoint information about cell viability. Hence, the advances in the use of prostate cancer organoids for screening new therapies is slow.

Objective: To address these shortcomings, we aimed to establish a scalable pipeline for automated seeding, treatment, and analysis of drug responses of prostate cancer organoids.

Study design and results: We established organoid cultures from five PDXs with diverse phenotypes of prostate cancer, including castrate-sensitive and castrate-resistant disease, as well as adenocarcinoma and neuroendocrine pathology. We robotically embedded organoids in 384-well plates, and monitored growth via brightfield microscopy prior to treatment with the PARP inhibitor talazoparib. Using bulk and single-organoid readouts of growth, including metabolic activity and live-cell imaging-based features, we showed that the responses of organoids to talazoparib were consistent with the sensitivity of each tumour *in vivo*. Single organoid analyses enabled in-depth assessment of morphological and compositional differences between patients and within organoid populations, revealing significant decreases in uniformity (Hoechst texture; $p<0.0001$) and density (Hoechst intensity; $p<0.0001$) in discrete subpopulations of PARPi-sensitive organoids.

Significance: In conclusion, we have demonstrated the ability to quantify changes in the growth of heterogeneous 3D cultures to candidate therapies across whole wells or specific subpopulations of organoids. By increasing the scale and scope of organoid experiments, this automated assay complements other patient-derived models and will expedite preclinical testing of new treatments for prostate cancer.

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Bipolar Androgen Therapy induces heterogeneous changes in the abundance and localisation of the androgen receptor in advanced prostate cancer

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Background: Androgen deprivation therapy is the primary treatment for advanced prostate cancer. Despite sustained suppression of androgens, patients eventually develop castration-resistant prostate cancer and require further treatment. A new

treatment is Bipolar Androgen Therapy (BAT), where patients rapidly cycle between castrate and supraphysiological testosterone levels to prevent tumours adapting to treatment. Clinical trials of BAT are promising, but patient responses vary.

Hypothesis & Aims: We hypothesised that changes in expression of the androgen receptor (AR) and truncated AR-variants (ARVs) were associated with response to BAT. To test this, we profiled AR and ARVs in patient-derived xenografts (PDXs) of prostate cancer before and after treatment with BAT.

Methods: We treated five, phenotypically diverse PDXs (1x BAT-sensitive, 4x BAT-resistant) with vehicle or BAT (fortnightly, intramuscular injections, 1mg testosterone cypionate). Tumours were harvested at 24-hours, 5 days, and endpoint (7-weeks or 1000mm^3). Expression of full-length AR (AR-FL), ARVs, and AR target-genes were measured using quantitative PCR. AR abundance and localisation was quantified using immunohistochemistry against different forms of the AR.

Results: Untreated PDXs had heterogeneous patterns of AR-FL and ARV expression. Acute BAT treatment down-regulated expression of AR mRNA, except in tumours with structural rearrangements of the AR gene (*i.e. 27.1, 27.2*). BAT also decreased nuclear AR protein levels in a BAT-sensitive PDX (*i.e. 201.1*), but not in BAT-resistant PDXs. Changes in AR-FL persisted at 5 days and endpoint, despite fluctuations in testosterone levels over a cycle of BAT. Expression of ARV mRNA mirrored differences in protein levels. Expression of PSMA, an AR target-gene, was suppressed over time, regardless of BAT response.

Conclusions: In patient-derived models, reduced nuclear AR protein expression following BAT may define a subset of tumours sensitive to BAT. BAT combination therapies may be an alternative for tumours that are resistant to BAT alone, and are currently in clinical trials.

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Establishment of neuroendocrine prostate cancer xenografts for preclinical testing

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Introduction: Neuroendocrine prostate cancer (NEPC) is an aggressive subtype of prostate cancer with a mean survival of less than 12 months after diagnosis. The poor prognosis of NEPC is partly due to its intrinsic resistance to androgen deprivation therapy. Thus, novel therapeutic strategies are urgently needed. While our understanding of the mechanisms regulating NEPC development is expanding, the translation of biological findings into clinical practice has been hampered by a lack of preclinical models that reflect the heterogeneity of this disease.

Objective: To address the shortage of preclinical models for NEPC, our goal was to establish a collection of patient-derived xenografts (PDXs) using tissues collected from men with prostate cancer.

Methods and results: Our laboratory has successfully derived 26 serially transplantable neuroendocrine PDXs from 12 patients across the clinical trajectory of prostate cancer. NEPC PDXs were histologically characterised by a pathologist and defined as positive immunohistochemical staining for one or more of the three neuroendocrine biomarkers: chromogranin A, synaptophysin, and CD56. Overall, we detected 6 of the 8 known histological variants of NEPC in this cohort, including small cell NEPC, large cell NEPC and amphicrine tumours. Interestingly, none of the three biomarkers were universally expressed in neuroendocrine PDXs. Next, by comparing NE biomarker expression to matched patient specimens, we confirmed that PDXs faithfully recapitulated the histopathology of original specimens despite years of serial transplantation. Finally, we attempted to broaden the preclinical capabilities of this collection by culturing organoids from 12 neuroendocrine PDXs. Overall, 10/12 lines were successfully grown as organoids; 4 of these lines achieved long-term, active growth in vitro and are suitable for preclinical testing.

Significance: In summary, we report a collection of 26 NEPC PDXs recapitulating the diverse histopathology of neuroendocrine tumours in contemporary patients and, therefore, is a valuable resource for examining the efficacy of novel compounds.

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Estrogen receptor β: a potential therapeutic target in adult granulosa cell tumours

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Ovarian granulosa cell tumours (GCT) express abundant estrogen receptor β (ERβ). Despite the high expression, ERβ signalling is transrepressed by the pro-survival transcription factor, NF-κB. Our preliminary data has suggested that activation of ERβ using selective estrogen receptor modulators (SERMs) may circumvent this transrepression. We hypothesise that the ERβ-specific ligands, diarylpropionitrile (DPN) and indazole, can circumvent NF-κB transrepression in KGN cells and subsequently activate ERβ signalling. In this study, we investigated the ability of the ER isoform-specific ligands DPN (100 nM), indazole (500 nM), bazedoxifene (10 nM), ethinyl estradiol (EE) (10 nM), and propyl pyrazole triol (PPT) (1 nM), to transactivate ER in a GCT-derived cell line (KGN). The endogenous physiological ER ligand 17β-estradiol (100 nM) was used as a control. KGN cells were transfected with estrogen-responsive reporter vector construct (ERE_2-luc) either alone or with ERα or ERβ expression vectors, to compare the effects of the individual ligands on endogenous and exogenous receptors. We found that the ligands had no effect on the endogenous ER, suggesting transrepression. However, we found induction of the reporter by exogenous ER with 17β-estradiol (3-fold (ERα); 7-fold (ERβ)), DPN (4-fold (ERα and ERβ)), indazole (3-fold (ERβ)), EE (9-fold (ERα); 8-fold (ERβ)), and PPT (5-fold (ERα); 8-fold (ERβ)). Interestingly, the highest activation of both exogeneous receptors, was by EE (9- and 8-fold

respectively), despite being widely known as only an ERα agonist. EE is commonly used in the oral contraceptive pill (OCP), has previously been shown to decrease granulosa cell mass in women who used EE-containing OCP. We speculate that these effects are likely to be ERβ-mediated due to this receptor being the predominant ER in these cells. These findings will add to our understanding on whether these ligands have a potential therapeutic use for ovarian GCT.

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Characterization of the kisspeptin and GnRH expression in the hypothalamus of the male and female taiep rat, an animal model of demyelination

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Reproductive alterations have been reported in patients with demyelinating diseases, these can lead to infertility. In the hypothalamus there are two neuronal types that regulate reproductive function; kisspeptin and GnRH neurons. At the present moment, the relation between demyelination and reproductive alterations is unknown; in order to elucidate this relation, this study used the taiep rat, which presents demyelination of the central nervous system along with reproductive issues. This study characterized the immunofluorescent expression of kisspeptin and GnRH in the hypothalamus of the male and female taiep rats using Sprague-Dawley rats as control. Brains of females during oestrous and adult males were cut at 10mm. Using immunofluorescence, the intensity of kisspeptin and GnRH in the hypothalamus was determined and analysed using the software ImageJ1.50i. Fluorescence is concentrated in fibre-like structures with neuron shape. An increment in the intensity of the fluorescence of kisspeptin was observed in male taiep rats compared with SD (0.37±0.02 vs 0.28±0.01, $p<0.001$). Female taiep rats during oestrus showed a decrease in the intensity of the fluorescence of kisspeptin structures compared with SD (0.44±0.02 vs 0.57±0.03, $p<0.001$). There were not any significant changes in the expression of GnRH fibres in the hypothalamus of the taiep rat compared with SD. Demyelination in the taiep rat affects kisspeptin expression at hypothalamic level, but this does not affect the expression of GnRH in either males or females, this effect is probably related with the availability of receptors in the GnRH neuron, this will be evaluated in the future. The decrease in kisspeptin expression in the female taiep rat could be caused by previously reported low concentrations of oestrogen, given that it is capable of stimulating kisspeptin expression.

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A pioneering approach to regulate steroid production using light.

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Steroid hormones regulate many crucial physiological processes including reproduction, response to stress, salt balance and various metabolic processes. Any alteration in their production or activity can have major pathophysiological implications such as cancers, endocrine disorders (ie polycystic ovary syndrome, Addison disease, hypogonadism, diabetes, stress) [1]. Hormone replacement therapy is the main treatment for steroid hormone deficiency, yet it involves daily administration and is associated with various short and long term risks. Thus, there is a recognised need to develop safer and more effective therapies to support steroid hormone production to maintain lifelong health. New technologies such as nano-implants inserted under the skin are being developed and could allow the assessment and controlled release of steroid with a single injection.

Steroid production (steroidogenesis) in the gonads/adrenals is regulated by the hypothalamic-pituitary axis. The Luteinising hormone (LH) and the adrenocorticotropic hormone (ACTH) bind respectively to their receptor (LHCGR) on the surface of Leydig cells and (MC2R) in adrenal cells. This activates the adenylate cyclase/cAMP production and regulate key enzymes in the steroidogenic pathway [2]. In this project, we developed a new way to control steroidogenic cell function using light-based system [3-4] bypassing the necessity of LH or ACTH binding to their receptors. We used a cell culture system to provide proof of concept that these optogenetic tools can efficiently activate LH and ACTH pathways, and induce endogenous steroid production. Our preliminary data in MLTC1 cells demonstrate that, following transfection of optogenetic construct, light (~470nm) exposure significantly stimulates steroid production. These novel findings provide the basis of a new technology that could be harnessed to develop new therapeutic strategies to control of steroid production, with the potential to transform the management of pathologies associated with steroid deficiency, with the likely benefits (reduced (i) side-effects, (ii) treatment burden and fine-tuned dosing regimens).

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Identification of novel antisense long non-coding RNAs in osteosarcoma: hidden gems

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The human genome encodes thousands of natural antisense long noncoding RNAs (lncRNAs); they play the essential role in regulation of gene expression at multiple levels, including replication, transcription and translation. Dysregulation of antisense lncRNAs plays indispensable roles in numerous biological progress, such as tumour progression, metastasis and resistance to therapeutic agents. To date, there have been several studies analysing antisense lncRNAs expression profiles in cancer, but not enough to highlight the complexity of the disease. In this study, we investigated the expression patterns of antisense lncRNAs from osteosarcoma and healthy bone samples (24 tumour -16 bone samples) using RNA sequencing. We identified 15 antisense lncRNAs (RUSC1-AS1, TBX2-AS1, PTOV1-AS1, UBE2D3-AS1, ERCC8-AS1, ZMIZ1-AS1, RNF144A-AS1, RDH10-AS1, TRG-AS1, GSN-AS1, HMGA2-AS1, ZNF528-AS1, OTUD6B-AS1, COX10-AS1 and SLC16A1-AS1) that were upregulated in tumour samples compared to bone sample controls. Further, we performed real-time polymerase chain reaction (RT-qPCR) to validate the expressions of the antisense lncRNAs in 8 different osteosarcoma cell lines (SaOS-2, G-292, HOS, U2-OS, 143B, SJSA-1, MG-63, and MNNG/HOS) compared to hFOB (human osteoblast cell line). These differentially expressed lncRNAs are potential therapeutic targets and biomarkers for osteosarcoma.

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Targetting mitochondrial dysfunction for the prevention of diabetic kidney disease

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Background:

Diabetic kidney disease (DKD) affects approximately one third of people with diabetes, greatly increasing the chance of cardiovascular disease and mortality. Current treatment strategies are limited to partially slowing down disease progression.

Mitochondrial dysfunction has been shown to be an important factor in the development and progression of DKD, with the high metabolic demands imposed by diabetes overwhelming the kidney's capacity to produce sufficient energy.

Here we propose that a portion of the people more susceptible to developing DKD may have underlying mitochondrial deficiencies and test the efficacy of a novel treatment designed to improve mitochondrial function, here named MitoA, in preventing the onset and/or severity or DKD in a mouse model of Type 1 Diabetes.

Methods

Mice, both wildtype and NDUFS6$^{+/-}$ (mitochondrial protein in electron transport chain), where induced with Type 1 diabetes (T1D) using streptozotocin and administered with "MitoA", a drug targeted at improving mitochondrial function. Blood glucose control was assessed by taking weekly BG levels, performing an oral glucose tolerance test (OGTT) and by measuring HbA1c levels. Kidney function/damage was assessed by measuring the glomerular filtration rate (via clearance of FITC-sinistrin), the albumin to creatinine ratio (uACR) and via histology (e.g., the glomerular sclerosis index).

Results

The diabetic mice with genetically exposed mitochondrial dysfunction (NDUFS6$^{+/-}$) had a decreased weekly area under the curve (AUC) for blood glucose levels (P=0.006) and for the OGTT blood glucose AUC (P=0.0006), with the treatment showing no statistically significant effect.

Conclusion

Increasing our understanding of the role of dysfunctional mitochondria in DKD progression may provide new, much needed therapeutics to halt the progression of kidney damage in people with diabetes.

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Differential gene expression analysis after PPARγ activation and XIAP inhibition identifies upregulation of metabolic processes in KGN cells

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Ovarian granulosa cell tumours (GCT) are hormonally active cancers characterised by indolent growth and late, invasive relapse. Aside from surgery the therapeutic options are very limited. We have previously reported a combination of activating the nuclear receptor peroxisome proliferator-activated receptor-gamma (PPARγ) and inhibiting the X-linked inhibitor of apoptosis protein (XIAP) as a potential specific therapeutic approach for GCT[1]. PPARγ, which impedes proliferation and promotes terminal differentiation in granulosa cells, is highly expressed in GCT. XIAP is also highly expressed in GCT. As a potent inhibitor of apoptosis, XIAP is an attractive therapeutic target. Combined XIAP inhibition and PPARγ activation results in regulation of several proteins involved in metabolic processes[2]. The GCT cells eventually undergo apoptosis[1]. We performed RNA-seq analysis on

GCT-derived KGN cells after combined PPARγ activation (rosiglitazone) and XIAP inhibition (Smac-mimetics). Generated RNA-seq libraries were sequenced (average of 50 million reads/sample). Data analysis utilised the RNASik pipeline (Monash Bioinformatics Platform), followed by DEGUST analysis to establish transcriptomic profiles. Preliminary analysis identified 165 differentially expressed genes in PPARγ-activated/XIAP inhibited KGN cells, 70% of which were upregulated (FDR 0.05, fold change>2). Gene Set Enrichment Analysis identified functionally related sets of genes involved in metabolic processes and cytokine-mediated signalling. We further validated the expression of two of the upregulated genes using RT-PCR. We found that PPARγ activation/XIAP inhibition caused a 12-fold upregulation of CC motif chemokine 20 (CCL20), a cytokine implicated in ovarian follicular cell differentiation. A 9-fold upregulation of Ras related glycolysis inhibitor and calcium channel regulator (RRAD) was also observed. RRAD is a GTP-binding protein and is associated with type II diabetes. Further work is being conducted to understand the basis of this upregulation. This study improves our understanding of the molecular mechanisms in GCT pathophysiology as well as enabling identification of new therapeutic targets.

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Platform for genome modification in human multipotent cell line

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Male sex is determined by expression of the Y chromosome gene *SRY* in the week 6 human XY embryonic gonad specifying its development into a testis; in the absence of SRY, the XX gonad develops into an ovary. Sex determination is a useful model to study cell fate decisions. The embryonic gonads are bipotential, and SRY differentiates the somatic cells into Sertoli cells that would otherwise become ovarian granulosa cells.

In about 1% of live births intersex conditions occur where babies are born with gonads typical of neither sex. The underlying molecular basis of intersex remains unknown in many forms[1], in part due to the lack of manipulatable models that recapitulate human sex determination. Mouse genetic models cannot be relied upon exclusively to recapitulate human intersex conditions, and show limitations such as functional redundancy, gene dosage or genetic buffering (alternative pathways existing for the same functional outcome) often resulting in no phenotypic consequence[2]. Furthermore, differences of gene expression thresholds and genetic robustness between humans and mice are becoming apparent[3].

A human *in vitro* model that can be used to model Sertoli cell function is NT2/D1, a multipotent clonal cell line derived from a testicular tumour[4]. NT2/D1 cells can model a variety of human developmental processes. Undifferentiated, they model early events in male sex determination, showing SRY activation of endogenous SOX9. Differentiated, they model neuronal development[5] or smooth muscle development[6] under different treatments.

We have established an NT2/D1-Cas9 cell line, and characterised these cells via a suite of cell phenotyping assays including xCELLigence® RTCA and HoloMonitor® live cell imaging. We will discuss their application to validate candidate intersex genes, deleted to assess their individual gene contributions to 'Sertoli cell like' characteristics such as cell adhesion, proliferation and migration, tight junction formation and germ cell maintenance.

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Perhexiline-associated hypoglycaemia

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Case

A 75-year-old female was admitted with severe hypoglycaemia (blood glucose 1.3mmol/L) and associated neuroglycopaenia. Relevant medical history included longstanding type 2 diabetes mellitus, end-stage renal failure secondary to diabetic nephropathy (on haemodialysis) and ischaemic cardiomyopathy.

She reported recent onset recurrent hypoglycaemia with blunted adrenergic symptoms. HbA1c was 6.7%. Risk factors for hypoglycaemia included exogenous insulin (insulin glargine 100u/mL 10 units nocte, insulin aspart 4 units pre-meal), renal impairment, reduced carbohydrate intake and perhexiline 100mg orally twice daily (commenced three months prior for angina). Retrospective review of subcutaneous insulin charts and blood glucose levels prior to perhexiline initiation did not reveal hypoglycaemia.

Exogenous insulin was ceased, and hypoglycaemia persisted for three days. This raised suspicion for possible perhexiline-associated hypoglycaemia. A perhexiline level was measured, with a result of 535ug/L (150-600), and OH-Perhexiline/Perhexiline ratio of 6.2ug/L, suggesting extensive metaboliser status. Notably, the perhexiline level measured ten days post-initiation was threefold lower (150ug/L). Plasma insulin and c-peptide were analysed on the hypoglycaemic venous sample and were mildly elevated at 14mU/L (2-23) and 2.7nmol/L (0.3-1.4) respectively, consistent with renal impairment.

Perhexiline was ceased, with improvement in appetite and resolution of hypoglycaemia two days later. FreeStyle Libre Flash Glucose Monitoring was used to monitor interstitial glucose post-discharge, with an average glucose of 9.1mmol/L. She subsequently recommenced basal insulin, with adequate glycaemic control and no ongoing hypoglycaemic burden (HbA1c 7.0%).

Discussion

Perhexiline inhibits mitochondrial carnitine palmitoyltransferase 1, shifting myocardial substrate metabolism from long-chain fatty acids towards glucose, increasing myocardial efficiency. Caution should be exerted when prescribing perhexiline to patients with diabetes on insulin or oral hypoglycaemic agents due to secondary changes in myocardial glucose utilisation and potential iatrogenic hypoglycaemia. Whilst the degree of glucose lowering does not necessarily correlate with perhexiline concentration, clinicians should remain vigilant for hypoglycaemia given perhexiline's polymorphic and highly variable metabolism.

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Associations between nutrients and foot ulceration in diabetes: a systematic review

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We reviewed the literature to evaluate potential associations between vitamins, nutrients, nutritional status or nutritional interventions and the presence or healing of foot ulceration in diabetes. Embase, Medline, PubMed, and the Cochrane Library were searched for studies published prior to September 2020. We assessed eligible studies for the association between nutritional status or interventions and foot ulcers. Fifteen studies met the inclusion criteria and were included in this review.

Six of the articles, by the same team of investigators, raised concerns due to the close similarities between recruited patients and highly similar outcomes across the set of studies. We are unsure whether this data is reliable. Of the other studies, 3 assessed vitamin D. In all three, vitamin D deficiency was common in participants with diabetic foot ulcers. One study reported 85% deficiency rates, another 97.1% and the last 55.7%. Vitamin A, B12, C and E and zinc deficiency are also more prevalent in foot ulcer patients.

Overall, there is a correlation between poor nutritional status and the presence of foot ulceration or a delay in healing. Because of heterogeneity, the studies were not suitable for meta-analysis. There is not enough data to reach conclusions about whether the relationships are causal. However, where a deficiency is identified for any vitamin or nutrient, it should be treated with dietetic and/or medical supervision. Randomised controlled trials are needed to investigate whether specific nutritional supplementation improves foot ulcer healing and reduces rates of lower limb amputation.

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An audit of emergency thyroidectomies for thyrotoxicosis: Local experience at Blacktown Hospital

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Objective: This audit aims to review cases of inpatient emergency thyroidectomies performed at Blacktown Hospital, including patient characteristics and post-operative complications.

Methods: The medical records of all patients who underwent thyroidectomy at Blacktown Hospital between June 2016 and August 2021 were reviewed. Operative indications, laboratory evaluation, pre-operative medications, clinical features (including Burch-Wartofsky Point Scale (BWPS)), histopathology and post-operative complications were determined for patients who underwent emergency thyroidectomy.

Results: Of the 181 patients who underwent total thyroidectomy at Blacktown Hospital, 9 (5%) were performed as emergency surgeries. 8 patients were female, and 1 patient was male, with an age range of 24-53 years. The predominant underlying pathology was Graves' disease (8 patients) and toxic multinodular goitre (1 patient). Pre-operative free T4 ranged between 19.2-52.5 pmol/L and free T3 ranged between 9.6-46.0 pmol/L. 8 patients received pre-operative Lugol's iodine and all patients received beta blockade. Indications for surgery included thionamide-related complications (6 patients) and uncontrolled thyrotoxicosis despite thionamide therapy (3 patients). Thionamide-related complications included severe pruritis and rash, leukopaenia, transaminitis, pancreatitis and febrile neutropaenia. The BWPS was calculated for each patient and ranged between 5-40 points, with the most common feature being tachycardia. There was no evidence of malignancy on histopathology. Post-operatively, symptomatic hypocalcaemia occurred in 2 patients and asymptomatic hypocalcaemia occurred in a further 2 patients, all of which were transient. All patients were vitamin D replete with a serum level > 50 nmol/L. Post-operative hypocalcaemia occurred in the 4 patients with the highest pre-operative free T4 and T3 levels amongst the cohort.

Conclusion: In our experience at Blacktown Hospital, the overall rate of emergency thyroidectomy is low. The most common indication for emergency thyroidectomy was thionamide intolerance and the most common underlying pathology was Graves' disease. Post-operative hypocalcaemia occurred in the patients with the more severe pre-operative biochemical hyperthyroidism.

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Diabetes complications and comorbidity screening in patients hospitalised with diabetes-related foot disease

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Background: Diabetes-related foot disease (DFD) is the leading cause of hospitalisation amongst people with diabetes. The Multidisciplinary Diabetes Foot Unit (MDFU) at Fiona Stanley Hospital provides interdisciplinary care for hospitalised patients with DFD. These patients also have significant risks of cerebrovascular, cardiovascular and renal disease and since 2019, we have adopted a simple written inpatient guideline with instructions on diabetes complication screening and the use of cardioprotective medications based on the American Diabetes Society Standards of Care. These guidelines were distributed to all members of the MDFU team including the rotating groups of junior doctors at the beginning of each term along with educational sessions on the implementation of these guidelines.

Aim: Our objective was to determine if the guideline improved screening for diabetes complications and the use of cardioprotective medications in patients admitted to MDFU.

Methods: We conducted an observational, retrospective study of patients admitted to MDFU comparing two 6-month periods, prior to and following the introduction of the guideline.

Results: 84 patients were studied in the first 6-month period and 138 patients were studied in the second 6-month period. HbA1c was measured in most patients in both phases (90.5% vs 95.7%, P=0.156). Following implementation of the guideline, there was no difference in screening of urine ACR (33.3% vs 21.0%, P=0.057) or lipid profile (53.6% vs 40.6%, P=0.071). Prescription of cardioprotective medications including statin therapy and renin-angiotensin aldosterone blockade was common in both groups but remained unchanged between phases.

Conclusion: Despite the introduction of the guideline, there was no improvement in opportunistic screening or use of cardioprotective medications in patients hospitalised with DFD, highlighting the challenges of inpatient screening. More research is required to identify strategies to improve opportunistic diabetes complication screening and cardiovascular risk management of this complex and high risk patient group.

Table 1. Demographic and clinical characteristics of patients admitted to MDFU at FSH before (Group 1) and after (Group 2) the introduction of the guideline

	Group 1	Group 2	p-value
Dates	1/9/2015 – 29/2/2016	1/9/2019 – 29/2/2020	
Number of patients	84	138	
Age (years)	62.5±13.2	61.9±13.3	0.742
Smoking status (%):			0.968
Never	36.9	38.4	
Ex-	39.3	38.4	
Current	23.8	23.2	
Type 2 diabetes (%)	86.9	89.1	0.669
Hypertension (%)	76.2	84.1	0.160
Dialysis (%)	1.2	1.4	>0.999
Hyperlipidaemia (%)	64.3	76.8	0.047
IHD (%)	36.9	21.7	0.020
Stroke (%)	14.3	4.3	0.011
HbA_{1c} checked at admission or within last 3 months (%)	90.5	95.7	0.156
HbA_{1c} (%)	8.3 [7.1-11.2]	9.4 [8.0-11.0]	0.062
uACR checked at admission or within last 6 months (%)	33.3	21.0	0.057
uACR (mg/mmol)	13.7 (1.5-125.5)	14.9 (2.6-87.5)	0.873
Lipids checked at admission or within last 6 months (%)	53.6	40.6	0.071
On statin (%)	72.6	71.0	0.878
On ACE or ARB (%)	61.9	65.9	0.565

Data are presented as percentages, mean±SD, median [IQR] or geometric mean (SD range).

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Screening for primary aldosteronism underutilised in a cohort of patients with chronic kidney disease

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Background

Primary aldosteronism (PA) is the most common and potentially curable endocrine cause of secondary hypertension, and carries a worse prognosis than essential hypertension. Despite the high prevalence of hypertension in patients with chronic kidney disease (CKD), the screening rates for PA in CKD are unknown.

Methods

In this study, we retrospectively reviewed medical records of 1627 adults with CKD who presented to the nephrology clinics of 2 tertiary hospitals in Melbourne, Australia, between January 2014 and April 2019. In addition to assessing the pattern of screening, we also evaluated patient-specific factors associated with the decision to test for PA.

Results

Of the 600 patients included in the final analysis, 234 (39%) had an indication for PA screening based on recommendations made by the Endocrine Society. However, only 33 (14%) were tested. They were younger (median age, 58 vs 72 years), had a higher mean systolic BP (153 vs 140 mmHg), better renal function (mean eGFR 51 vs 37 mL/min/1.73m^2), and lower mean serum potassium (4.1 vs 4.5 mEq/L) than those who were indicated but not screened. Of the 33 screened patients, an elevated ARR was noted in 8 patients and a diagnosis of PA was made in 4 patients.

Conclusions

The screening rate for PA is low in a CKD population, especially in patients who are older, have a lower eGFR and normal serum potassium. The consequences of undiagnosed PA in this select population may be substantial due to the cardiovascular and renal sequelae associated with untreated disease.

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Outcomes of an integrated care approach for patients at our multidisciplinary weight management clinic extending through COVID-19 initial lockdown.

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An integrated approach is needed to achieve durable weight loss and improvements in metabolic health, that is not only anthropometric-centric but also as a chronic disease complication-centric. The last 18 months with the COVID-19 pandemic has challenged care provision and the ability to optimise weight management for society overall. The literature indicates a consistent finding of adverse outcomes with COVID-19 in individuals with the metabolic syndrome.

Aim: To evaluate the results of patients receiving individualised multidisciplinary care from specialists in endocrinology, dietetics, exercise physiology, and surgery at the Healthy Weight Clinic (HWC).

Methods: A retrospective chart review was conducted to identify patients who attended an initial consultation at HWC between March 2017–March 2019 and followed up through the pandemic thus far. Changes in weight, BMI, waist circumference, percentage weight loss, and body composition outcomes and obesity related complications were evaluated. Analysis during the 2020 lockdown was also performed.

Results: 199 of the total 239 patients followed up at the HWC and were treated by the multi-specialist team (83.3% reattendance rate). Average baseline weight was 106.9kg(21.2kg SD) and BMI 37.9kg/m2(7.1 SD). Of patients who reattended the clinic the mean final weight change was -9.0kg(11.1 SD) equating to -7.9% mean body weight change. With multidisciplinary support patients maintained weight loss during the pandemic or with re-engagement with clinical care were able to re-achieve weight loss. 38.4% of patients who returned reduced their BMI class by at least one class and improved their obesity related comorbidities, improving their stage of obesity.

Conclusion: Multi-specialist holistic care provides clinically significant weight loss with better outcomes in the patients regularly following up with the team both in the medical and surgical sub-cohorts, especially during the COVID-19 pandemic. Our results also demonstrate that there is an improvement in metabolic co-morbidities with holistic care.

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The impact of a revised DKA protocol on the prevalence of hypokalaemia in a tertiary teaching hospital: a before-and-after study

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Objectives: Hypokalaemia is a potential complication of diabetic ketoacidosis (DKA). We assessed the impact of a revised DKA protocol on the prevalence of hypokalaemia during treatment at our tertiary centre.

Methods: Retrospective audits were conducted on adult patients (≥18 years) with DKA (pH<7.35, blood glucose>11mmol/L and capillary ketones>1.0mmol/L) admitted between Jan 2016 – December 2016 (cohort 1) and September 2020 – July 2021 (cohort 2) following implementation of a revised DKA protocol with a faster intravenous potassium replacement rate on the wards.

Results: Cohort 1 consisted of 59 patients (mean age 43.5±20.4yrs; 88% type 1 and 12% type 2 diabetes) with 68 episodes of DKA. Cohort 2 included 58 patients (mean age 42.8±20.8yrs; 83% type 1 and 17% type 2 diabetes) with 68 DKA episodes. Hypokalaemia (serum potassium <3.5mmol/L) occurred in 5.9% at presentation in cohort 1 and 2.9% in cohort 2 (*P*=0.408). In the first 48 hours of treatment, 54.4% and 38.2% developed hypokalaemia in cohorts 1 and 2, respectively (*P*=0.103). Hypokalaemia was mild (3.0-3.4mmol/L) in 39.7% vs 34.6%, moderate (2.5-2.9mmol/L) in 10.3% vs 6.7%, and severe (2.0-2.4mmol/L) to critical (<2.0mmol/L) in 4.4% vs 0% (*P*=0.568). Intensive care unit (ICU) admission occurred in 36.8% in cohort 1 (median ICU length of stay (LOS) 2 days) and 41% in cohort 2 (median ICU LOS 1 day). In both cohorts, median hospital LOS was 3 days, resolution of DKA within 24 hours (pH >7.3 with capillary ketone <1.0mmol/L) occurred in >90%, and no arrhythmia or in-hospital mortality was observed.

Conclusions: The introduction of a revised DKA protocol appears to have mitigated the rate of severe and critical hypokalaemia during treatment. However, the prevalence of hypokalaemia during treatment remains high, with a 10 to 15-fold increase in the first 48 hours, highlighting the need to remain vigilant of this complication.

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Hypophosphataemia in association with the treatment of diabetic ketoacidosis: predictive significance of severity of acidosis at presentation

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Objectives: Hypophosphataemia is commonly associated with the treatment of diabetic ketoacidosis (DKA). However, literature on the dynamics of serum phosphate and determinants of hypophosphataemia in DKA remains scarce. We evaluated the change in serum phosphate during DKA treatment and assessed whether acidosis severity on admission was related to the degree of hypophosphataemia.

Methods: A retrospective review of adult patients (aged ≥18 years) with DKA (pH<7.35, blood glucose >11mmol/L and capillary ketones >1.0mmol/L) admitted between September 2020—July 2021. Those without a serum phosphate (Pi) at presentation or serial Pi during DKA treatment were excluded.

Results: Of 75 DKA episodes, 36 in 28 patients, mean±standard deviation (SD) age 45.4±20.2yrs, 75% type 1 and 25% type 2 diabetes, met inclusion criteria. At presentation, 58.3% were hyperphosphataemic (Pi>1.50mmol/L). Initial Pi (mean±SD 1.68±0.61mmol/L) correlated with initial serum glucose (r =0.522, *P*=0.001), but not serum creatinine (r =0.185, *P*=0.281). Pi decreased during treatment in all cases (mean±SD Pi reduction 1.19±0.67mmol/L, mean±SD nadir Pi 0.49±0.25mmol/L), with a hypophosphataemic (<0.75mmol/L) nadir Pi in 83.3% and severe hypophosphataemia (<0.32mmol/L) in 16.7%. Initial serum bicarbonate correlated with nadir Pi (r =0.582, *P*<0.001). Using linear regression, every 1.0mmol/L decrease in serum bicarbonate was associated with an average reduction of 0.05mmol/L in Pi. Mean±SD initial bicarbonate differed between those with a nadir Pi <0.5mmol/L and ≥0.5mmol/L (8.1±4.6mmol/L vs 13.1±6.3mmol/L, *P*=0.022), whereas those with severe hypophosphataemia had a mean±SD bicarbonate of 7.8±4.4mmol/L vs 10.7±6.0mmol/L (*P*=0.270) compared with those with nadir Pi ≥0.32mmol/L. No adverse effects of hypophosphataemia on morbidity or mortality were noted.

Conclusion: There is a significant relationship between the degree of metabolic acidosis at presentation and the extent of hypophosphataemia during DKA therapy. Clinicians should be vigilant of the risk of severe hypophosphataemia during DKA treatment in patients with profound acidosis, particularly in those with a serum bicarbonate of <10mmol/L.

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Patient satisfaction and endocrine and metabolic effects of estetrol, a novel estrogen, in combination with drospirenone for contraception

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OBJECTIVE: To assess patient satisfaction and metabolic effects of a new oral contraceptive containing estetrol (E4) 15mg and drospirenone (DRSP) 3mg.

BACKGROUND: E4 is an estrogen exclusively produced by the human fetal liver. E4 plus DRSP demonstrated high contraceptive efficacy and an excellent safety profile in two phase-3 trials performed in Europe/Russia and North America.

METHODS: Data is derived from two separate 6-cycle, phase-2 studies in healthy participants.

E4/DRSP (n=79) participants provided data pertaining to well-being and satisfaction (Subject Satisfaction and Health-Related Questionnaire) in an open-label, multi-centre, dose-finding, parallel study with estradiol valerate (E2V)/dienogest (DNG) (n=78) as reference.

The impact of E4/DRSP (n=38) on endocrine and metabolic parameters was evaluated in a randomized, open-label, controlled, 3-arm, parallel study compared with ethinylestradiol (EE) 30µg/levonorgestrel (LNG) 150µg (n=29), or EE 20µg/DRSP 3mg (n=31). Median percentage changes from baseline to cycle 6 were assessed.

RESULTS: Overall well-being scores for E4/DRSP were comparable with E2V/DNG. At cycle 6, 73.1% of subjects were (very) satisfied using E4/DRSP vs. 67.6% subjects using E2V/DNG. The number of women willing to continue with the assigned study treatment was the highest in the 15mg E4/ DRSP group (82.1%)

Compared with EE/LNG and EE/DRSP, E4/DRSP induced less pronounced changes in cortisol (+26.0% vs. +109.0% and +107.0%), cortisol binding globulin (+40.0% vs. +152.0% and +140.0%), thyroxine binding globulin (+17% vs. +37% and +70%) and angiotensinogen (+75.0% vs. +170.0% and +206.5%). E4/DRSP had minimal lipid effects, with the largest effect on triglycerides (+24.0%), similar to EE/LNG (+28.0%) and less than EE/DRSP (+65.5%). E4/DRSP had no effect on carbohydrate metabolism and TSH remained relatively stable in all treatment groups. No clinically relevant changes in blood pressure or pulse rate were observed.

CONCLUSION: E4/DRSP is associated with a high-user satisfaction. Compared to EE-containing contraceptives, E4/DRSP has limited effects on endocrine and metabolic parameters.

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'If I have another fall, I could break my leg' - the experience and perspectives of patients with non-hip minimal trauma fractures

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Purpose: While personal impact and patient experience following a hip fracture is well documented, the patient illness experience following non-hip minimal-trauma-fractures (MTFs) (e.g.: distal radius, vertebral and humeral) is rarely studied. We sought to explore the patient experience and expectations of fracture care in an acute tertiary hospital for non-hip MTF

patients. Understanding the patient experience and journey from fracture to recovery, can help enable implementation of patient-centered care post MTFs.

Methods: Interpretive phenomenological analysis (IPA) was adopted, which is well suited to explore how participants make sense of their world and experiences. Participants were recruited from the Liverpool Hospital Osteoporosis Refracture Prevention Clinic. Semi-structured interviews exploring illness experience and impact of the fracture were conducted until thematic saturation was reached.

Results: Twelve women and 3 men aged from 59 to 79 years old were recruited in the study until thematic saturation was reached. Four themes were identified through the patient interviews in their experience post fracture: Fear of another fracture, impact of the fracture, gratitude for the service and behaviour change post fracture.

Conclusion: The impacts of hip fractures are well documented in the literature, however, the impact of non-hip MTFs and the impact on the patient as a consequence remains to be investigated. Our findings assist in understanding the patient experience of those individuals who have sustained a non-hip MTF. There is a need for providing education, pharmacological treatment options, exercise and dietary advice, emotional and social support; and addressing patient's post-fracture pain.

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Interpretative dance: Improving the clinical utility of IGF-1 based on locally derived reference intervals

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Background

IGF-1 has long been a difficult analyte to measure, with well documented assay shifts in 2012. It became apparent that our current reference intervals (RI) were too high in the paediatric population and too low in the adult population. Issues around insufficient age partitioning in the paediatric population were also present.

Aims

To establish improved RIs for IGF-1, based on data from the local West Australian population. Assess the total variability (both biological and analytical) of IGF1.

Methods

IGF- 1 results between 2016-20, performed in PathWest laboratories, were assessed with gross outliers removed from the data. The remaining top and bottom 5 centiles were removed, leaving results that lie between the 5^{th} and 95^{th} centiles. Within the data set, patients with no history of pituitary disease or growth hormone disorder, who had 3 of more serial IGF1 tests done were identified, and analysed to assess total variability in IGF1.

Results

The resulting dataset contained 6943 results from 4230 patients. This data was used to derive a refence interval using local data as well as centile based continuous reference intervals. This data set is far larger than the 1500 patients used to derive the existing reference interval (as per kit insert) [1]. Within the data set, 91 patients with 301 results were identified as having no pituitary disease and had serial IGF1 measurements as part of an annual cardiovascular risk assessment. These results were used to assess IGF 1 variability.

Conclusion

Use of local data with a large number of patients and results is likely to provide a more reliable reference interval for our local population. Continuous centile based reference intervals may help identify patients whose results remain within the reference interval but whose centile has changed markedly.

Table 1

Age	New IGF-I RI (derived from local data)	Immulite IGF-1 RI (from kit insert)
20 to <25	**113 – 407**	116 - 358
25 to <30	**100 – 380**	117 – 329
30 to <35	**87 - 353**	115 – 307
35 to <40	**87 – 327**	109 – 284
40 to <45	**80 – 327**	101 - 267
45 to <50	**80 – 287**	94 – 252
50 to <55	**73 – 287**	87 - 238
55 to <60	**67 – 273**	81 - 225
60 to <65	**67 – 273**	75 - 212
65 to <70	**60 – 273**	69 - 200
70 to <75	**60 - 273**	64 - 188
75 to <80	**53 – 260**	59 - 177
80 to <85	**47 – 233**	55 - 166
85 to <90	**47 – 220**	-
90 to <120	**40 – 207**	-

Table 1 demonstrates the difference between reference intervals derived from local data (in bold) and those provided by the kit insert.

1. Siemens Immulite 2000 IGF-1 reagent kit insert

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Risk stratification of indeterminate thyroid nodules using ultrasound and machine learning algorithms

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Background: Indeterminate thyroid nodules (Bethesda III) are challenging to characterise without diagnostic surgery. Auxiliary strategies including molecular analysis, machine learning models and ultrasound grading with TI-RADS can help to triage accordingly, but further refinement is needed to prevent unnecessary surgeries and increase positive predictive values.

Design: Retrospective review of 88 patients with Bethesda III nodules who had diagnostic surgery with final pathological diagnosis.

Measurements: Each nodule was retrospectively scored through TI-RADS. Two deep learning models were tested, one previously developed and trained on another dataset, mainly containing determinate cases and then validated on our dataset while the other one trained and tested on our dataset (indeterminate cases).

Results: The mean TI-RADS score was 3 for benign and 4 for malignant nodules (p=0.0022). Radiological high risk (TI-RADS 4, 5) and low risk (TI-RADS 2,3) categories were established. The PPV for the high radiological risk category in those with >10mm nodules was 85% (CI 70-93%). The NPV for low radiological risk in patients >60years (mean age was 100% (CI 83-100%). The AUC value of our novel classifier was 0.71 and differed significantly from the chance-level (p=0.0009).

Conclusions: Novel radiomic and radiologic strategies can be employed to assist with pre-operative diagnosis of indeterminate thyroid nodules.

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Inpatient Hypoglycaemia: Not A Benign Event. A Case Control Study

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Introduction:

Approximately 7.5% of adult Australians have diabetes mellitus (DM); 25% of hospital inpatients have DM. Inpatient hypoglycaemia occurs frequently but its significance is under recognised.

Aims:

To determine the incidence of and predictors for inpatient hypoglycemia, and the association of hypoglycaemia with mortality and hospital length of stay (LOS) in an Australian tertiary hospital.

Methods:

All patients admitted to Nepean Hospital over 12 months from July 2018 to June 2019 who experienced an inpatient hypoglycaemic event (HE), defined by capillary blood glucose level<4mmol/L, were included. HE patients were assigned to two groups: those with a diagnosis of DM and those without. The control group comprised randomly selected inpatients admitted over the same time period with a DM diagnosis but no HE.

Data extracted from electronic medical records (eMR) included: age, gender, length of stay (LOS), diabetes type, kidney injury, serum albumin, nutritional state dietitian assessed), presence of active malignancy, HbA1c, BMI, hypoglycaemic medications, hypoglycaemic awareness, medical team documentation of HE, in-hospital mortality and 12-month mortality.

Results:

251 hypoglycaemic events were identified: 202 (80%) in patients with DM and 49 (20%) without diabetes. 200 patients with DM without a HE formed the control group.

Risk factors for a HE included being malnourished (p=0.03), basal-bolus insulin use (p<0.01), mixed insulin use (p=0.01) and sulphonylurea use (p=0.03).

In patients with DM, comparing those with and without HE: median LOS was three-fold higher (12 versus 4 days), mean in-patient mortality was 3 fold higher (10% versus 3%) and mean 12-month mortality was 4-fold higher (22.5% versus 5.5%). Patients without DM with HE had a 34% 12-month mortality.

Conclusion:

Inpatient hypoglycaemia is common and is associated with a trebling of inpatient mortality, hospital length of stay and a 4-fold increase in 12-month mortality. Further research into preventative measures is urgently needed.

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Assessment of Glycaemic Profiles 24 hours following Exercise of Different Intensities in Individuals with Type 1 Diabetes

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Aims

Preventing post-exercise hypoglycaemia in individuals with Type 1 diabetes (T1D) is challenging with no clear guidelines to prevent hypoglycaemia after exercises of different intensities. The aim of this study was to explore glycaemic profiles over 24 hours following exercise of different intensities in individuals with T1D in a free-living setting.

Methods

This study was a secondary analysis of data collected in individuals with T1D, as part of a larger lab-based four-arm randomised counterbalanced study examining the carbohydrate requirements to maintain euglycaemia for different exercise intensities. Eight participants with T1D (mean±SD age 25.1±5y; HbA1c 7.9±0.8%) receiving continuous subcutaneous insulin infusion, were tested on four separate occasions, on cycle ergometers for up to 40 min at four exercise intensities (VO_{2peak} of 35%,50%,65%,80%). Data from continuous glucose monitoring system for 24 hours post-exercise were analysed using paired t-tests and Wilcoxon signed-rank tests. The outcomes of this study were the percentage of time spent in range (TIR) and rate of prevented or actual hypoglycaemic events in the 24 hours post-exercise.

Results

The percentage of TIR at 35%, 50%, 65% and 80%VO_{2peak} were 50.77±23.01%, 60.44±17.74%, 53.55±23.91% and 57.15±15.07% respectively with no statistical differences across intensities being detected ($p \geq 0.05$). The mean rate of prevented or actual hypoglycaemic events/day for exercise at 35%, 50%, 65% and 80%VO_{2peak} were 3.01 (95% CI: 1.72, 4.88), 3.13 (95% CI: 1.89, 4.94), 2.51 (95% CI: 1.29, 4.38) and 3.43 (95% CI: 1.96, 5.58) respectively with no differences between groups.

Conclusion

There was no difference in hypoglycaemic events 24 hours following exercise of four intensities. The small sample size and missing data might have diminished the ability of the study to detect effects of exercise. Our data can be used for further research to design an adequately powered study to determine the effect of exercise intensity on glycaemic control.

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COVID-19 and Diabetes: A 1 year Follow-Up

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Introduction:

"Long COVID" has been used in the literature to describe persisting symptoms in patients surviving severe COVID-19. Interim result of the Australian prospective study (the ADAPT study) showed 40% of the participants had ongoing fatigue, shortness of breath, and chest tightness after 9.8 weeks of diagnosis. The impact of COVID on diabetes long term is currently unknown.

Objective:

We previously characterised 8 patients with type 2 diabetes admitted to Westmead Hospital ICU with COVID-19 between 20 March and 1 May 2020. For this study, we aim to assess the impact of COVID-19 infection on glycaemic control of these patients 6-18 months after their initial COVID-19 diagnosis.

Methods:

We followed up on the glycaemic control and diabetes therapy used 6-18 months post COVID-19 infection. Medication list was confirmed through documentation in the COVID follow-up clinic. HbA1cs were obtained through the hospital and external pathology laboratories.

Results:

Eight out of nine patients with type 2 diabetes were admitted to Westmead Hospital ICU with COVID-19 in 2020. Mean HbA1c was 8.9 % at admission. All patients required high insulin doses during their peak inflammatory response. 6 to 18 months post COVID-19 infection, their mean Hba1c was 7.5%. Only one patient was on insulin prior to admission however four patients were discharged on insulin therapy. At time of analysis, only one patient remained on insulin therapy. Patients who were on oral hypoglycaemic agents at time of admission had not required escalation of therapy in this study. All patients had follow-up pulmonary function test and four patients had reduced total lung capacity. Their FEV1/FVC and diffusing capacity of lung were within normal limits.

Conclusion:

Our study showed no evidence of detrimental effects of COVID affecting their glycaemic control. Analysis on a bigger cohort of COVID patients in future is warranted for further investigation.

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Phaeochromocytoma- a single centre experience

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Background and Aims

Phaeochromocytomas are rare, highly vascularised neuroendocrine tumours arising from the chromaffin cells of the adrenal medulla[1]. These catecholamine secreting tumours occur in less than 0.2- 0.6% of patients with hypertension[1]. Serious cardiovascular morbidity and mortality is associated if left untreated. Standard approach involves pharmacological management of blood pressure and subsequent surgical resection. Despite treatment, these individuals are at long term risk of metastases and disease recurrence.

The aim of this study is to describe the prevalence, clinical spectrum, biochemical profile, preoperative management, surgical and longer term outcomes of phaeochromocytoma patients at Lyell McEwin Hospital, a tertiary centre in South Australia.

Methodology

Retrospective review of case records of histologically proven cases of phaeochromocytomas from 2010 to 2021. Data presented will include demographics, symptomatology, history of hypertension, history of known adrenal lesion, investigations, time from diagnosis to surgery, peri operative blood pressure control, intra and post operative complications and follow up post surgery.

Results

Phaeochromocytoma was histologically diagnosed in 13 patients from 2010 to 2021. 11 were surgically operated on and 2 are awaiting surgery. 3 of the 11 cases operated on have since died. Further data is currently being analysed and demographics, investigations, complication rates and post operative progress will be presented in detail.

Conclusion

Through the comparison of our findings with available guidelines and published data from other single centres internationally, this will assist with the development of a protocol for the preoperative management and standardisation of care of phaeochromocytoma patients in our centre.

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An emphasis on the increased morbidity and mortality in elderly patients with thyroid cancer undergoing thyroidectomy – a meta-analysis study

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Thyroid cancer diagnoses are the third fastest rising cancer diagnosis in the USA alone. Despite thyroid cancer patients generally having an excellent prognosis, it has been shown that elderly patients are more likely to undergo sub-therapeutic management, despite having more aggressive disease. This study addresses the risk of mortality and morbidity in elderly patients undergoing thyroid surgery for thyroid cancer. This study quantitatively investigated the risks of elderly patients who underwent thyroidectomy for thyroid cancer regarding mortality/survival, recurrence of disease, and complications arising from thyroidectomy. A systematic search and meta-analysis of journal articles was carried out using the electronic databases PubMed and Medline. These articles contained epidemiological evidence of mortality and recurrence of disease in patients within two groups: above the age of 60, and below the age of 60; who are treated for operatively thyroid cancer and data involving complications following total thyroidectomy were included in the meta-analysis for this study. The meta-analysis consisted of a total of 16 studies which met the inclusion and exclusion criteria. This study confirmed that patients have increased risk of recurrence (HR 4.84; 95% CI = 2.22-10.52; I2=0.00; P=0.98) including increased risk of lymph node recurrence and distant metastases. Additionally, there was an increased risk of complications (OR 1.82; 95% CI = 0.88-3.77; I2 =77.01; P = 0.005) following thyroidectomy compared to patients in the younger cohort. This study also qualitatively compared survival data between the two age cohorts, and identified a reduced overall survival and disease free survival for elderly patients. This study puts forth evidence for the classification of elderly patients as higher risk of mortality and morbidity following total thyroidectomy for thyroid cancer and puts further emphasis on early detection and intervention.

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Current Trends in Thyroid Cancer Diagnoses in Australia

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The incidence of thyroid cancer has been previously reported to be on the rise around the world. A study in the USA had shown a tripling of diagnoses between 1975 and 2009[1]. It is thought to be due to better detection of small primary cancers because of improved ultrasound techniques. This study aims to determine if the trend of increasing incidence of thyroid cancer continues in Australia. This study used data cubes obtained from the Australian Institute of Health and Welfare (AIHW) and were analyzed to assess trends in thyroid cancer diagnosis over the period from 1982-2017. Results of the analysis shows an approximate 10-fold increase in the incidence of thyroid cancer over 35 years. 361 Cases in 1982 compared with 3154 cases in 2017. The number of thyroid cancer cases was predicted to rise to 3830 new cases in 2021. Incidence and mortality in Australia are higher than the

WHO standards. Despite this, mortality per case has been shown to be decreasing over time. Operative management of has shown a preference toward hemithyroidectomy over total thyroidectomies overall. The current projections in the AIHW data cubes do not consider the impact that the SARS-COV-19 virus on the health care system and flow on effect for thyroid cancer diagnoses in Australia [2]. This consideration will likely cause a significance reduction in the number of new cases diagnosed and the trends of mortality and operative management are likewise expected to be affected. This study provides reference trends of incidence, mortality, operative management of thyroid cancer in Australia over an interval of 35 years.

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Quality of Life of patients with Primary Aldosteronism treated with MR antagonists compared to ENaC inhibitors

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Measurement of Quality of Life (QOL) forms an integral part in assessing disease and treatment impact from the patient's perspective. While prior studies compare the QOL of patients with primary aldosteronism (PA) undergoing surgical management compared with pharmacotherapy, there is little published data comparing different pharmacological regimens and effect on QOL. Their varying mechanism of action, side effect profile and efficacy may contribute to different QOL outcomes. An online survey was distributed through international PA patient support groups to assess patient demographics, baseline health status and QOL. Using the validated 36-Item Short-Form Health Survey (SF-36), as well as a PA-specific questionnaire, we compared the QOL outcomes of patients with PA treated with mineralocorticoid receptor antagonists (MRA, spironolactone or eplerenone) and patients treated with epithelial sodium channel inhibitors (ENaCi, amiloride). Seventy-nine patients with medically-managed PA (73 taking MRA, 82% female; 6 taking ENaCi, 100% female; mean age 36 years) completed the survey. There were no significant differences in the body mass index, blood pressure, total number of medications and total number of comorbidities between the treatment groups. SF-36 scale scores of patients taking MRA tended to be greater in the domains of role-emotional and mental health compared to those taking ENaCi (62.1 vs 44.4, 61.6 vs 53.3) but lower in the domain of general health (37.5 vs 52.5). However, the differences did not reach statistical significance. The PA-specific QOL questionnaire tended to demonstrate higher QOL in the domain of "fluid balance" in patients taking MRA, without being statistically significant (6 vs 8). No significant difference was observed in SF-36 domains or PA-specific questionnaire between patients taking spironolactone or eplerenone. This study demonstrated trends towards differences in specific QOL domains between patients treated with MRA or ENaCi. A larger sample size is required to ascertain the statistical significance of these differences.

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Glycaemic control and pregnancy outcomes in a multicultural cohort of women with type 1 diabetes

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Aim: To assess the glycaemic control and adverse pregnancy outcomes in a multicultural cohort of women with type 1 diabetes.

Method: An audit of the Diabetes in Pregnancy service at Blacktown Hospital was undertaken to identify women with type 1 diabetes between 2010-2020. Data was acquired from the Electronic Medical Record for demographics, trimester specific HbA1c, treatment approach, and adverse pregnancy outcomes. The primary outcome was to evaluate the proportion of women meeting optimal glycaemic control according to ADIPS guidelines (HbA1c ≤ 6.5% in 1st trimester, ≤6.0% in 2nd and 3rd trimesters). Secondary outcomes included the assessment of adverse maternal outcomes (pregnancy loss, pre-eclampsia, pre-term labour) and neonatal outcomes (macrosomia, SGA/IUGR, neonatal hypoglycaemia, respiratory distress), and comparison between women utilising fingerprick or continuous (CGM) glucose monitoring.

Results: Data on 66 pregnancies were analysed. The mean HbA1c in the 1st, 2nd and 3rd trimesters were 7.6%, 6.6% and 6.9% respectively with 26%, 17% and 14% of the cohort achieving respective trimester specific glycaemic targets. A total of 102 adverse pregnancy outcomes occurred (44% maternal, 69% neonatal) in the cohort (Table 1). Pre-term delivery (32%), macrosomia (28%), and neonatal hypoglycaemia (48%) were the most common events. Nineteen women (29% of cohort) utilised CGM. Glycaemic control based on HbA1c was similar between the CGM and non-CGM groups at each trimester time point. Macrosomia occurred less frequently in the CGM (16%, n=3) vs non-CGM group (33%, n=14), with other outcomes similar between groups.

Conclusion: The achievement of tight glycaemic control in pregnancies complicated by type 1 diabetes remains a challenge with glycaemic targets achieved in only a subset of women. Adverse pregnancy outcomes remain occur frequently in this high-risk group of women.

TABLE 1 – GLYCAEMIC CONTROL AND ADVERSE OUTCOMES

HbA1c (target per trimester)	Mean ± SD (%)	Number in target (% of total)
▪ 1st trimester (≤6.5%)	7.6 ± 1.5	13 (25.5%)
▪ 2nd trimester (≤6.0%)	6.6 ± 0.9	10 (16.7%)
▪ 3rd trimester (≤6.0%)	6.9 ± 0.8	7 (13.0%)
Adverse outcomes	**Number**	**% of pregnancies affected**
Neonatal hypoglycaemia	29	47.5
Preterm delivery (< 37 weeks)	20	32.3
Macrosomia	17	27.9
Neonatal respiratory distress	15	24.6
Pre-eclampsia	10	16.1
SGA/IUGR	6	9.8
Pregnancy loss	5	7.6
Total adverse outcomes	**102**	**84.8**
▪ Maternal	35	43.9
▪ Fetal/Neonatal	67	69.4

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An eight year audit on hypertriglyceridaemia-induced pancreatitis management

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AIMS

Hypertriglyceridaemia is a rare cause of acute pancreatitis (AP). It is worsened by alcohol excess and poorly controlled diabetes mellitus (DM). There are no recommended guidelines for management, which leads to poor treatment. This audit reviews the management of hypertriglyceridaemia-induced AP over eight-years.

METHODS

A two-centre retrospective study was conducted for AP over 8.75years. Management of those with elevated triglyceride levels (defined as ≥6mmol/L) on admission was reviewed. Data was collected from electronic records.

RESULTS

Between 01-Nov-2011 and 31-Jul-2020, there were 3850 presentations of AP to either Gold Coast Hospital or Robina Hospital. 75 presentations (1.94%) between 51 patients had elevated triglycerides during admission (mean 52.4 ±47.9mmol/L). 81.3% had documentation that hypertriglyceridaemia was the major contributing cause, whilst the rest were documented as secondary to acute alcohol ingestion despite elevated triglycerides.

Average length of hospitalisation was 8.45days often under general surgical team. Average patient was 41.92years with BMI of 30.4. All were fasted with IV fluids with 32% requiring insulin infusion and 9% needing plasma exchange. 22.6% required intensive care admission. 4% required surgical intervention. There was 1 death, due to concomitant sepsis.

In the 42 cases where family history was obtained, diabetes mellitus (21.4%) and hyperlipidaemia (64.2%) were common. Pre-existing diabetes mellitus affected 50.7%, however HbA1c was measured in only 38.7% (mean 8.73% ±3.07%). There was a high recurrence of AP, with 58.6% having either prior or future episodes.

Endocrinologist opinion was obtained in 42.7% and dietician review in 48%. Only 76% left hospital on pharmacological therapy.

CONCLUSION

Whilst hypertriglyceridaemia is a rare cause of AP, it can be mismanaged with lack of specialist or dietician opinion, and patients can be discharged without pharmacotherapy. This increases recurrence leading to worsening morbidity.

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Beware small thyroid nodule not just what but where

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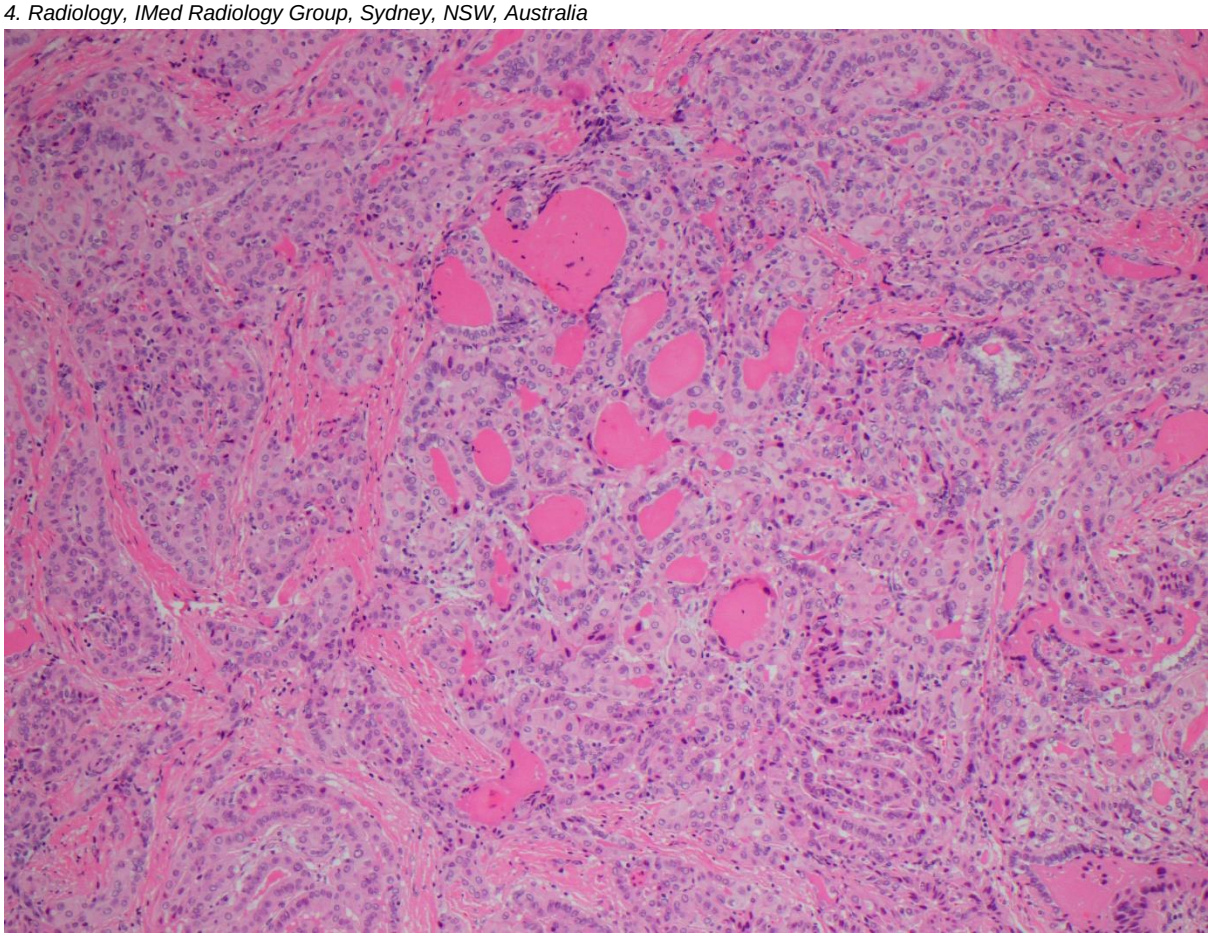

A 33 year-old female working as an IT professional and part time soprano was worked up for weight management. Medical history included asthma and an elevated BMI at 33Kg/m^2. Subclinical hypothyroidism was identified with a TSH of 6.0 mIU/L (0.4-3.5) and T4 of 14 pmol/L (9-19). A palpable thyroid gland prompted ultrasound which demonstrated a 9 x 7 x 6mm left mid-pole nodule abutting the trachea. It was taller than wide, very hypoechoic, with an irregular margin and TIRADS of 5. FNA biopsy was consistent with papillary thyroid carcinoma (BETH 6). Surgical review raised concern of the proximity of the nodule to the trachea. CT demonstrating no discernable fat plane between the nodule and trachea. A complete thyroidectomy was performed without complication. Histopathology showed an 8mm tumour with lymphovascular invasion in the inferior pole. It had follicular architecture and extrathyroidal extension at the level of the recurrent laryngeal nerve, which was surgically spared. Multiple other foci were seen throughout the gland. One 2.5mm lymph node deposit was found contralateral to the primary tumour. This case raises the importance of appraising the features of each thyroid nodule including location. ATA guidelines may not have prompted biopsy of this sub-centimetre nodule, and a delay in treatment would have been associated with far greater morbidity in particular invasion of the recurrent laryngeal nerve, trachea and further metastasis.

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Opioid induced androgen deficiency in an outpatient clinic

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Background

Opioid induced androgen deficiency (OPIAD) is prevalent among opioid users. Testosterone replacement therapy (TRT) can improve quality of life, body composition and pain sensitivity[1]. We sought to differentiate clinical characteristics of OPIAD patients with other hypogonadal males.

Method

Audit was performed on adult male patients diagnosed with hypogonadotropic hypogonadism (early morning testosterone <10mmol/L, FSH and LH in low-normal range) in a general endocrinology clinic. Exclusion criteria: primary hypogonadism, pituitary disorder, PSA>4ng/mL and malignancy. Data was analysed using SPSS expressed as mean+SEM and statistical significance determined at p<0.05.

Results
17 of 46 patients used opioids for >3 months. Buprenorphine (35%) was most common opioid followed by Targin (24%). Baseline characteristics were as follows:

	Age (years)	Weight (kg)	Baseline testo level (nmol/L)	PSA (ng/ml)	Prolactin (mIU/L)	IGF-1 (mIU/L)	Cortisol (nmol/L)	Haematocrit
Opioid (n=17)	59.7±4.2	103.3±7.1	4.0±0.4	0.6±0.3	287±79	19.8±2.7	443±99	0.40±0.01
Non-opioid (n=29)	50.2±2.6	105.4±2.3	6.5±0.4	0.8±0.2	273±61	25.0±1.6	300±40	0.45±0.01
P-value	0.06	NS	0.006	NS	NS	NS	NS	0.003

Main indication for opioid use was musculoskeletal pain (96%) and mean morphine equivalent daily dose (MED) was 141+60mg. MED correlated inversely with baseline testosterone (p=0.006). Opioid users reported more fatigue (100%) but less libido (12%) and erectile dysfunction (12%) compared to non-opioid users (p<0.01). Hypogonadal symptoms were unrelated to opioid-type when testosterone, weight, and MED were considered. Anaemia (Hb<130g/L) was significantly associated with opioid use (11.8% vs none) (p<0.01). There were no significant associations with other co-morbidities (e.g. osteoporosis, diabetes, cardiovascular disease, depression).

Thirty-nine patients received intramuscular testosterone undecanoate (Reandron). Treatment led to rise in haemoglobin and testosterone levels. It was also associated with symptomatic improvement and reduced self-reported pain.

Conclusion
OPIAD is under-recognised, and highly prevalent. In this audit, opioid users had significantly lower baseline testosterone levels and lower haematocrit values. All opioid users complained of fatigue and a significant proportion had mild anaemia. TRT leads to symptomatic improvement and increased testosterone levels.

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General practitioner perspectives and experiences when screening for primary aldosteronism in hypertensive patients

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Background:
Primary aldosteronism (PA) is a common form of hypertension caused by autonomous production of aldosterone independent of renin. Screening with an aldosterone renin ratio enables early detection and targeted treatment, which can reduce cardiovascular complications(1). However, screening rates are low among Australian general practitioners (GPs)(2). Limited awareness is thought to explain the low screening rates in general practice(3).

Objective:
To understand the factors that influence a GP's decision to screen for primary aldosteronism.

Method:
We used a qualitative study to explore the experiences of GPs when screening for PA. Set in South-East Melbourne, participating GPs received an educational session on PA from an endocrinologist. We conducted semi-structured interviews with GPs who had screened at least one patient following the teaching session. Interviews were transcribed, independently coded, and analysed for emerging themes.

Results:
The 16 GPs varied by clinical experience (1-35 years), practice location (regional, urban), and number of patients screened for PA (1-44). GPs preferred screening newly diagnosed hypertensive patients. Only a few GPs opted to screen all hypertensive patients, while most questioned the necessity of screening patients whom they thought fitted their clinical impression of essential hypertension. Many GPs found it challenging to both comply with testing requirements and interpret screening results amidst the organisational constraints of their practice. GPs that had diagnosed at least one case of PA acknowledged the significant impact it had on patient wellbeing and this reinforced their role in assisting with the detection of PA. Knowledge, cost, and convenience of the screening process, conceptualisation of risk and perceived impacts of detecting PA influenced the screening experience.

Conclusion:
This study demonstrates that additional factors, other than limited awareness, influence GP screening decisions. Our findings have the potential to inform future policy, practice, and training to improve the detection of PA in Australian general practice.

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A young woman with childhood onset bone pain and late diagnosis of Hereditary Hypophosphatemic Rickets with Hypercalciuria

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Hereditary hypophosphatemic rickets with hypercalciuria (HHRH) is a rare autosomal recessive condition. Implicated genes produce missense mutations which disrupt sodium-phosphate co-transport, resulting in renal phosphate wasting[1 2]. Clinically HHRH causes nephrolithiasis /calcinosis, bone pain, rickets, lower extremity deformities and short stature[1].

We report on a 43-year-old lady with symptoms from childhood, with complex osteomalacia, limb deformities & rickets. Through to young adulthood, she had multiple bilateral femoral shaft osteotomies and surgeries. She migrated to Australia in her late 20s and was referred to endocrinology with initial management of intravenous bisphosphonates for chronic vertebral insufficiency fractures seen on imaging. Prior to the correct diagnosis, biochemistry serially showed low to normal phosphate, normal to mildly suppressed PTH, markedly elevated 1,25 hydroxyvitamin D levels($1,25(OH)_2D$), bone turnover markers in the upper range of normal to mild elevation; 24-hour urine tests showed intermittent hypercalciuria and hyperphosphaturia. Serum fibroblast growth factor 23 (FGF23) levels were low on both measurements.

Estimated prevalence of HHRH is 1:250,000[2]. Unlike the more common X-linked hypophosphatemia, it is a FGF23-independent disorder[2]. HHRH is marked by renal phosphate wasting and appropriately elevated $1,25(OH)_2D$ levels, which in turn increase intestinal calcium absorption and reduce PTH-dependent calcium-reabsorption in the distal renal tubules. Clinically this causes hypercalciuria and other manifestations.

Our index patient is one of eight children of consanguineous Iraqi & Syrian parents, but the only member affected. Due to clinical suspicion of a hereditary bone syndrome, she was referred to clinical genetics and a homozygous (pathological) variant within the SLC34A3 gene with missense mutation on both alleles eventually confirmed.

HHRH is rare, so underrecognized with diagnosis often delayed[2]. Current treatment recommendation is long-term phosphate supplements[2]. Active vitamin D metabolites are contraindicated, as it worsens hypercalciuria[2]. For our patient, high dose phosphate supplementation alleviated all her residual bone symptoms.

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Aldosterone, renin and ARR variability in the detection of primary aldosteronism

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Background: The plasma aldosterone concentration (PAC), renin and aldosterone-renin ratio (ARR) are used to screen for primary aldosteronism (PA). A recent study (Yozamp et al. Hypertension. 77: 891, 2021) reported substantial intraindividual variability of PAC and ARR (based on plasma renin activity) in the context of usual antihypertensive therapy. The intraindividual variability of PAC and direct renin concentration (DRC), a more widely used measurement of renin, in the absence of interfering medications is unknown but important for the interpretation of a single ARR performed to screen for PA.

Method: In this retrospective study of patients who attended an Endocrine Hypertension Service from May 2017 to July 2021, those with at least two ARR results off interfering medications were analysed. PA was formally diagnosed using the seated saline suppression test following an abnormal ARR > 70 pmol/L:mU/L. Variability in PAC and DRC was calculated as coefficient of variation (CV = standard deviation / mean) and percent difference (difference between highest and lowest values / mean).

Results: The final analysis included 223 patients (55% females), with a median age of 52 years. Significant variability was seen in both PAC and DRC with CV of 24% and 41%, and percent difference of 45% and 75%, respectively. No significant differences in CV or percent difference were seen between patients with or without PA, and in patients with different subtypes of PA. Forty-five out of the 180 patients with PA (25%) could have had a missed diagnosis if the ARR had not been repeated.

Conclusion: Intraindividual variability in PAC, DRC and hence ARR occurs in a significant number of patients being investigated for PA. Findings from our study advocate for the measurement of at least two ARR, particularly if the first ARR is normal, before discounting the potential diagnosis of PA.

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Outcomes of nodule evaluation in a thyroid nodule and cancer clinic

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We audited endocrinologist consultations on patients attending a referral clinic for assessment and review of thyroid nodules and thyroid cancer follow-up, between January 2018 and December 2019. 309 patients were seen: established thyroid cancer (121), clinically-apparent nodules (104), and incidental nodules (84), with mean age 59 years (226 female). 195 patients with all data available in the electronic medical record were analyzed: established thyroid cancer (45), clinically-apparent nodules (78), and incidental nodules (72). Ultrasonography (US) was analyzed by Thyroid Imaging Reporting and Data System (TIRADS) and cytopathology by the Australian Modified Bethesda Criteria (AMBC).

After initial assessment, 154 nodules in 118 individuals underwent cytological examination: 12 AMBC-I, 72 AMBC-II, 12 AMBC-III, 14 AMBC-IV, 8 AMBC-V, 36 AMBC-VI. 41 patients with 58 nodules (4 AMBC-I, 11 AMBC-II, 1 AMBC-III, 7 AMBC-IV, 5 AMBC-V, 30 AMBC-VI) proceeded to thyroidectomy with 48 nodules showing malignant histology. A further 15 patients underwent hemithyroidectomy for 16 nodules with 14 being malignant (6 AMBC-III, 2 AMBC-IV, 1 AMBC-V, 5 AMBC-VI), i.e. 62/154 (40%) of initially biopsied nodules were malignant. Results for 36 nodules (27 individuals) resulted in discharge from follow-up (2 AMBC-I, 33 AMBC-II, 1 AMBC-IV with poor prognosis from additional malignancy).

Of remaining patients, after one further US, biopsy was performed for 31 nodules in 25 patients, with subsequent thyroid surgery in seven patients and histologically-confirmed malignancy in five (surgery for compressive symptoms in three). Continued follow-up for a mean of 48 months in the remaining patients identified one further malignancy.

We conclude 1) there was a high yield of malignancy in those submitted to surgery; 2) there is a very low rate of malignancy in those not submitted to surgery after the initial or one subsequent assessment, over 4 years follow-up; 3) criteria for serial follow-up should be revised to reduce unnecessary review.

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Microwave ablation for primary hyperparathyroidism and its effect on bone metabolism in 20 patients

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Object To investigate the efficacy and safety of ultrasound-guided microwave ablation (MWA) in the treatment of primary hyperparathyroidism (PHPT) and evaluate MWA effect on bone metabolism. **Methods** A total of 20 PHPT patients were treated by MWA in our center from May 2019 to June 2021. The changes of serum parathyroid hormone (PTH), calcium and phosphorus were observed before and after ablation. The markers of bone metabolism, renal function, the volume as well as volume reduction rate of parathyroid lesion were compared before treatment and the last follow-up thereafter. The technical success, complete and partial clinical success rates of treatment were recorded as well. **Results** At the last follow-up, the median serum PTH and calcium levels in 20 patients decreased significantly compared with that before ablation ($P<0.05$). A complete response for both PTH and calcium levels was achieved in 11 of 20 patients, while 7 patients had slightly elevated PTH level only above the upper limit of normal reference range. The total clinical cure rate was 90%. The median level of 25(OH)D at the last follow-up was significantly higher than that before ablation ($P < 0.01$). The median level of total procollagen type I N-telopeptide as well as β-crosslaps determination was significantly lower than that before ablation ($P < 0.01$). There was no detectable change in renal function during the follow up period. The ablated volumes were all significantly decreased than before ($P<0.05$), and the technical success rate was 66.7%. No serious complications were observed. **Conclusions** Ultrasound-guided MWA is safe and effective in the treatment of PHPT and can improve bone metabolism.

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Feminising hormone therapy regimens and cardiovascular risk factors in older transgender individuals

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Introduction: The safety and efficacy of feminising hormone therapy in older transgender (trans) individuals is unclear. Current recommendations suggest transdermal estradiol beyond the age of 45 years, especially if cardiometabolic risk factors are present. We aimed to evaluate feminising hormone therapy regimens and cardiovascular risk factors in older trans individuals.

Methods: A retrospective cross-sectional analysis was undertaken of trans individuals attending a primary or secondary care clinic in Melbourne, Australia who had received estradiol for at least six months duration. Serum estradiol concentration was measured by immunoassay. Outcomes were: (1) feminising hormone regimens and serum estradiol concentrations by age group: (a) ≥45 years, (b) <45 years, and (2) prevalence of cardiometabolic risk factors in individuals ≥45 years.

Results: 296 individuals were stratified by age group: ≥45 years (*n*=55) and <45 years (*n*=241). There was no difference in median serum estradiol concentration between groups (328 pmol/L vs. 300 pmol/L, p=0.22). However, there was a higher proportion of individuals ≥45 years treated with transdermal estradiol (31% vs. 8%, p<0.00001). Of those treated with oral estradiol, the median dose was lower in the ≥45 years group (4mg vs. 6mg, p=0.01). The most prevalent cardiometabolic risk factor in the ≥45 years group was hypertension (29%), followed by current smoking (24%), obesity (20%), dyslipidaemia (16%) and diabetes (9%).

Conclusions: A greater proportion of trans individuals ≥45 years of age were treated with transdermal estradiol. Of those who received oral estradiol, the median dose was lower. This is important given the high prevalence of cardiometabolic risk factors in this age group, however cardiovascular risk management guidelines in this patient cohort are lacking.

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A retrospective review of testosterone treatment in adult men with Prader-Willi syndrome

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Background: Hypogonadism is the most frequent hormonal deficiency in individuals with Prader-Willi syndrome (PWS). This often necessitates testosterone treatment, but limited data are available to guide testosterone treatment in adult men with PWS. The aim of this audit was to evaluate the safety, tolerability and efficacy of testosterone treatment in individuals with PWS.

Methods: A retrospective audit was undertaken of individuals with PWS attending the Austin Health Weight Control Clinic between July 2010-April 2021. Main outcome measures were testosterone formulation and dose, serum total testosterone concentration, and adverse effects.

Results: Data were available for 8 individuals with mean age 28.4 years (range 21-58) with BMI 44.8 kg/m^2. Six men had obstructive sleep apnoea; none were smokers. Baseline total testosterone concentration was 2.6 nmol/L with haematocrit 0.43. Men were treated with testosterone for mean 6.0 years (range 1-12). Formulations were intramuscular (IM) testosterone undecanoate (TU) 1000mg (n=5), transdermal testosterone gel 50mg daily (n=1), and oral TU 80-120mg daily (n=2). Mean (SD) total testosterone concentration was 11.0 (4.5) nmol/L. Testosterone concentrations within the male reference range were only achieved with IM TU. Mean haematocrit during treatment was 0.47(0.04) with 9 (36%) >0.50. Three individuals with haematocrit >0.52 were treated with IM TU 1000mg every 12 weeks, with trough serum total testosterone 8.8-15.9 nmol/L. One individual had haematocrit >0.54 that necessitated decreased frequency of IM TU. IM TU was well tolerated whereas one individual discontinued testosterone gel due to poor compliance. Worsening behavioural disturbance with physical aggression resulted in treatment discontinuation in one individual, with subsequent improvement in behaviour.

Conclusions: Intramuscular TU achieved adequate serum testosterone concentrations and appears well tolerated in men with PWS but was complicated by polycythaemia, reinforcing the need for regular monitoring. Families and carers should be aware of the risk of worsening behavioural disturbance with testosterone treatment.

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A retrospective review of intensive weight loss interventions for treatment of obesity in people with Prader-Willi syndrome

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Background: Prader-Willi syndrome (PWS) is characterised by hyperphagia with childhood-onset obesity. Strict dietary supervision and restriction is integral to prevent weight gain, but limited data are available to guide intensive weight loss interventions (VLED, pharmacotherapy, bariatric surgery) in this population. The aim of this study was to evaluate the safety, tolerability and efficacy of intensive weight loss interventions in individuals with PWS.

Methods: A retrospective audit was undertaken of individuals with PWS attending the Austin Health Weight Control Clinic between July 2005-April 2021. Main outcome measures of intensive weight loss interventions (VLED, pharmacotherapy) were duration of use, weight outcomes, and adverse effects.

Results: Data were available for 18 individuals, of whom 14 were treated with intensive weight loss interventions. Mean body weight at baseline was 96.8 kg (BMI 40.8 kg/m^2). Mean weight loss during VLED (n=7) was 11.7 kg over 132 weeks, though did not result in weight loss in two individuals. Combination pharmacotherapy was most commonly prescribed. Mean weight loss with phentermine-topiramate (n=7) was 16.2 kg over 56 weeks. Mean weight loss with liraglutide 0.6-3mg (n=7), prescribed with topiramate in 3 individuals, was 14.9kg over 138 weeks. Weight loss was documented in 5 of 7 individuals treated with liraglutide. Naltrexone-bupropion resulted in weight loss in 2 of 4 individuals. Five individuals discontinued pharmacotherapy due to adverse effects (phentermine: psychosis, insomnia; topiramate: rash, depression, memory impairment). Five individuals maintained >10% weight loss at last follow-up. Non-adherence with prescribed regimen resulted in weight regain; mean weight at last follow-up was 98.4 kg (BMI 41.3 kg/m^2). No individual underwent bariatric surgery.

Conclusions: VLED and pharmacotherapy can be successfully utilised in some individuals with PWS though non-adherence results in substantial weight regain. Adverse effects were ascribed to phentermine and topiramate and resulted in discontinuation, whereas liraglutide was well-tolerated in this population.

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Prescription patterns and testosterone concentrations achieved with AndroForte 5 testosterone cream for trans and gender diverse individuals

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Background: Masculinising hormone therapy with testosterone is used to align an individual's physical characteristics with their gender identity. Standard testosterone doses and formulations recommended for cisgender hypogonadal men are typically administered, though there are no data evaluating the use of AndroForte 5 testosterone cream in gender-affirming hormone therapy regimens.

Aims: To assess the prescription patterns and serum total testosterone concentrations achieved with AndroForte 5 testosterone cream in trans and gender diverse individuals.

Methods: A retrospective longitudinal analysis was undertaken of trans individuals at a primary and secondary care clinic in Melbourne, Australia. Seventy-two individuals treated with AndroForte 5 testosterone cream were included. Primary outcomes were testosterone dose and serum total testosterone concentration achieved.

Results: Median age was 26 years (IQR 22-30) and median duration of testosterone therapy was 14 months (7-24). Fifty-two (72%) individuals had a non-binary gender identity. Initial mean (SD) testosterone dose was 70 (30) mg daily. Thirty-eight (53%) commenced doses <100mg daily, the recommended starting dose for hypogonadal cisgender men. Median total testosterone concentration achieved from 186 individual laboratory results was 11.1 nmol/L (7.5-15.9). Polycythaemia was documented in 9 of 171 (5%) laboratory results.

Conclusions: AndroForte 5 testosterone cream achieves serum total testosterone concentrations within the male reference range and represents an alternative route of testosterone administration for trans and gender diverse individuals seeking masculinisation. A high proportion of individuals had a non-binary gender identity, with over 50% commencing a lower dose than that administered to hypogonadal cisgender men, potentially related to slow or partial masculinisation goals.

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Effects of micronised progesterone on sleep, psychological distress and breast development in transgender individuals: a prospective case-control study

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Background: The role of micronised progesterone in hormone regimens for transgender (trans) individuals undergoing feminising hormone therapy remains uncertain, though there are anecdotal reports of improved mood and enhanced breast development. We hypothesised that micronised progesterone would improve sleep quality, psychological stress and breast development in trans individuals on established feminising hormone therapy.

Methods: We conducted a three-month prospective, observational case-control study of 23 trans individuals newly commencing 100mg micronised progesterone and 19 controls continuing standard care therapy. Outcome measures included (i) Pittsburgh Sleep Quality Index (PQSI); (ii) Kessler psychological distress (K10) scale; and (iii) Tanner stage. A linear mixed model was used to compare mean differences between groups.

Results: Compared to controls over 3 months, there was no difference in PSQI [mean difference 0.163 95% CI (-1.86, 2.19) *P*=0.87), K10 [1.129 95% CI (-1.68, 3.94) *P*=0.43] or Tanner stage [0.126 95% CI (-0.22, 0.47) *P*=0.46]. There was no statistically significant difference in the proportion of individuals with clinically significant improvements in PSQI (25% vs. 17%, *P*=0.70) or K10 (20% vs. 11%, *P*=0.66). One individual had a significant deterioration in psychological distress that improved following cessation of micronised progesterone.

Conclusions: Micronised progesterone was not associated with changes in sleep quality, psychological distress or breast development over three months follow-up, though there was significant inter-individual variability. Larger, placebo-controlled trials are required to further evaluate different doses of micronised progesterone in feminising hormone therapy regimens.

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Craniopharyngioma surgical resections and their management: a single centre experience from 2010 to present

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Background/Aim

Craniopharyngiomas are benign, invasive tumours. Given their low incidence, contemporary data about treatment outcomes are limited. The Royal Melbourne Hospital (RMH) is a quaternary centre for adults with pituitary disease that performs a high volume of pituitary surgery, hence we investigated our experience over the last decade.

Methods

We audited outcomes for patients who underwent craniopharyngioma resection at RMH from 2010-2021, including: surgical approach, perioperative complications, residual disease, radiotherapy, diabetes insipidus (DI), hypopituitarism, hypothalamic dysfunction and mortality.

Outcomes

Eighteen patients underwent craniopharyngioma resection, performed by one surgeon. Common presenting complaints included visual disturbance (56%), hypopituitarism (39%) and headache (22%). The median age at diagnosis was 43 years (16-71). Six (33%) were diagnosed aged 16-20 and 9 (50%) aged 48-71, a bimodal distribution. Two underwent craniotomy and the remainder had an endoscopic endonasal transsphenoidal approach.

Fifteen (83%) had recurrence/residual disease; 6 underwent further surgery and 9 received radiotherapy. Thirteen have been stable on serial imaging.

Permanent vision loss was present in 2 (11%) patients. Two, age 66 and 76 are deceased, both with other comorbidities at the time of death.

DI occurred in 14 (78%) patients and was transient in 3 (21%). 3 (16%) were reported to have hypothalamic dysfunction. Seventeen (94%) had at least partial hypopituitarism post operatively; all requiring glucocorticoid and thyroxine replacement. Twelve (67%) patients required oestrogen/testosterone therapy and 2 (11%) were treated with growth hormone (GH) replacement.

Discussion

Our contemporary series resembles the limited published data, with a mean age of 36-43 years and primary presenting complaints of visual disturbance (67-75%) and hypopituitarism (42-60%)[1]. Post-operative rates of DI and hypopituitarism were also comparable. GH treatment rates were low, suggesting many may not have been tested for GH deficiency since PBS GH became available. The reported incidence of hypothalamic dysfunction was low with a modern surgical approach.

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Optimizing Dynamic StudieS in EndocrinologY (ODYSSEY): An Endocrine nurse initiative - Preliminary data

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Background

The Royal Melbourne Hospital (RMH) is a tertiary/quaternary centre for the management of adult endocrine and pituitary diseases. Until May 2021, endocrine dynamic investigations have been coordinated by the Endocrine registrars, however increasing demand for dynamic investigations, and competing clinical commitments for the registrars was limiting availability of these tests. Therefore, in May 2021 we implemented measures to optimise dynamic investigations including:

- Training an Endocrine Grade 4b Registered Nurse to perform and coordinate reporting of dynamic investigations (0.4 FTE).
- Formalising protocols for dynamic investigations
- Formalising presentations of dynamic investigation data by the Endocrine registrar to 2 Endocrinologists at a fortnightly meeting.

Aim

To determine if Endocrine nurse led endocrine dynamic investigations improve patient outcomes at RMH.

Methods

Dynamic investigations performed between May-July 2021 were compared to the corresponding period in 2020. Outcomes measured included: number of dynamic investigations performed and resultant treatment changes. Baseline and 3-month satisfaction scores for Endocrinology registrars were also assessed (completely dissatisfied (1)-completely satisfied (5)).

Results

At baseline (May-July 2020), 9 dynamic tests were performed including 3 glucagon stimulation tests (GSTs) and 1 adrenal vein sampling procedure (AVS) (Table 1). Two patients had adult growth hormone deficiency (AGHD) and one commenced GH replacement. The patient who had AVS was managed medically. Following intervention (May-July 2021), 21 dynamic tests were performed, including 9 GSTs and 3 AVS. Seven had AGHD; Four received GH treatment education from the endocrine nurse, two have planned education and one was enrolled in a GH clinical trial. Three AVS procedures demonstrated unilateral primary hyperaldosteronism in two patients, who were referred for adrenalectomy. Staff satisfaction improved from 2/5 to 4/5.

Conclusions

Endocrine nurse led dynamic investigations have improved testing rates, treatment and staff satisfaction. Ongoing funding for an Endocrine nurse is paramount for providing optimal care for patients with pituitary disease.

Table 1: Endocrine dynamic investigations performed pre and post intervention			
	May-July 2020	May-July 2021	Change
Glucagon stimulation test (GST)	3	9	+3-fold
Saline suppression test (SST)	2	4	+2-fold
Adrenal vein sampling (AVS)	1	3	+3-fold
Mixed meal tests (MMT)	0	4	> +4-fold
Insulin tolerance test	0	1	> +1-fold
Water deprivation tests	2	0	> -2-fold
Inferior petrosal sinus sampling (IPSS)	0	1	> +1-fold
Dexamethasone CRH test	1	1	No change
Total	9	23	+2.6-fold

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The Identification and Management of Adult Growth hormone INsufficiEncy (IMAGINE): an Endocrine nurse initiative - Preliminary data

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Background

Royal Melbourne Hospital (RMH) is a quaternary centre for management of adult pituitary disease. Growth hormone (GH) replacement is now available on the PBS, however GH commencement is resource intensive: screening for eligible patients, performing a GH stimulation test, completing the QoL-AGHDA questionnaire and educating patients about treatment. Historically, this has been coordinated by the Endocrinology registrars. In May 2021, we introduced an Endocrine nurse initiative to improve the detection and management of adult GH deficiency (AGHD). The initiative consisted of appointing and training an Endocrine Grade 4b RN to screen our Pituitary Database for potential patients, coordinate GH stimulation testing and provide GH treatment education.

Aim

To determine if an Endocrine nurse initiative improves the detection and management of AGHD at RMH.

Methods

We audited the number of GH stimulation tests performed and the number of patients diagnosed with AGHD who commenced GH therapy from May-July 2021 compared with the same period in 2020. Baseline and 3-month process satisfaction scores for Endocrinology registrars were also assessed (completely dissatisfied (1)-completely satisfied (5))

Results

At baseline (May-July 2020), 3 glucagon stimulation tests (GSTs) were performed and 2 patients had AGHD; one commenced GH therapy. After introduction of the Endocrine nurse (May-July 2021), 9 GSTs were performed and 7 had AGHD. Four patients received GH treatment education from the endocrine nurse, 2 have upcoming education appointments and 1 was enrolled in a GH clinical trial. The introduction of the Endocrine nurse improved satisfaction scores for the Endocrine Registrars from 2/5 at baseline to 4/5 at 3 months.

Conclusions

The introduction of an Endocrine nurse to improve the detection and management of AGHD has improved rates of testing, diagnosis, treatment and staff satisfaction. Ongoing funding for an Endocrine nurse is paramount for providing quaternary level care for patients with pituitary disease.

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A mixed insulin regimen can address glucocorticoid-induced hyperglycaemia

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Aim: Prednisolone is widely used for its anti-inflammatory and immunosuppressive properties, but is frequently accompanied by hyperglycaemia. There are limited data to guide selection of an insulin regimen for treatment of glucocorticoid-induced hyperglycaemia (GIH), and data are specifically lacking on the use of mixed insulin in GIH. We hypothesised that mixed insulin (NovoMix30®) for GIH as a daily (mane) or twice-daily (mane + midi) regimen would provide effective glycaemic control while minimising nocturnal hypoglycaemia.

Methods: We performed a retrospective analysis of adult inpatients at a tertiary centre who were co-prescribed prednisolone ≥7.5mg mane and NovoMix30® mane +/- midi for >48 hours from November 2018 to June 2020. Blood glucose levels (BGLs) were retrieved for 54 patients across four days, beginning from the day before insulin commencement (day 0). A subgroup (n=37) received methylprednisolone ≥ 48 hours before oral prednisolone commencement. The expectation-maximisation algorithm was used to impute missing BGL values (30.7%) and repeated measures analysis was used to compare BGLs across days and between subgroups.

Results: The prednisolone-only subgroup had higher mean HbA1c ($p<0.0001$), initial prednisolone dose ($p<0.05$) and BGLs across all time windows ($p<0.001$). There was no significant interaction of steroid type with time of day or days of treatment, so results were pooled. NovoMix30® significantly decreased glucose levels in the morning ($p=0.001$) and afternoon ($p=0.019$) without an effect on fasting or evening levels (Figure 1). However, glycaemic control was suboptimal overall with insulin doses of ≤0.22 U/kg and insulin/prednisolone ratios of ≤0.39 U/mg. One hypoglycaemic event (BGL 3.7 mmol/L) occurred among 69 BGLs recorded from midnight to fasting.

Conclusion: Although limited by its retrospective nature and incomplete BGL data, this study demonstrates that mixed insulin mane +/- midi targets the glucose profile seen with GIH, however higher doses are required.

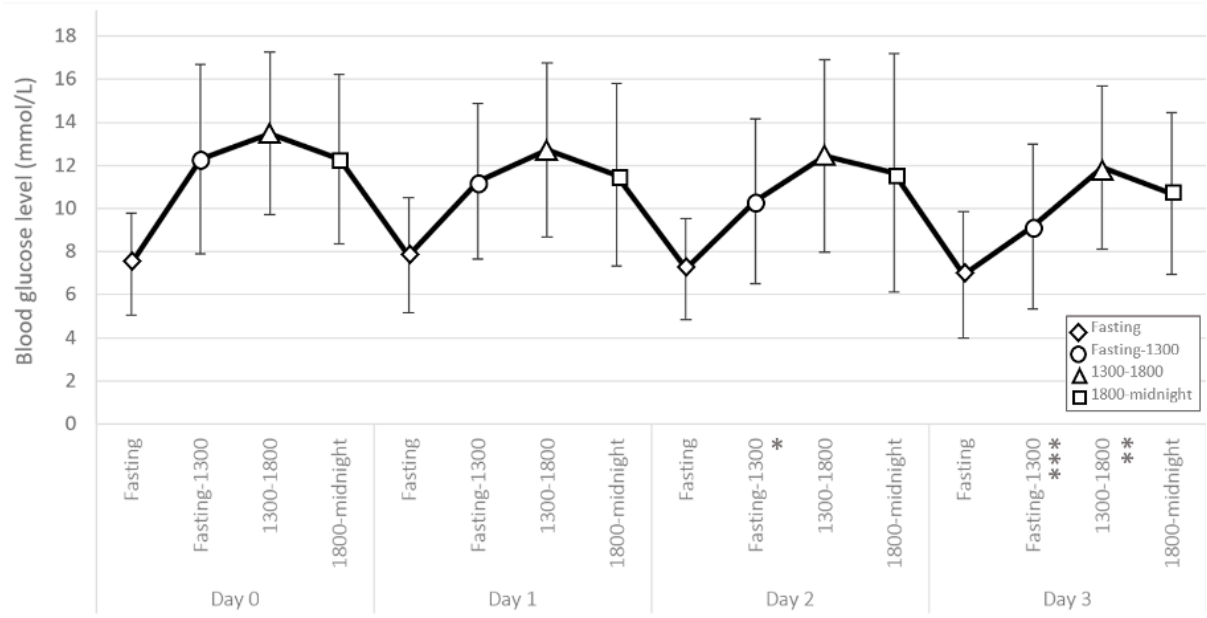

Figure 1: BGLs (mean ± SD) with the use of NovoMix30® for the management of glucocorticoid-induced hyperglycaemia from days 1-3. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for comparison to day 0.

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Risky business: calculated cardiovascular risk underestimates real risk in hypertensive patients with primary aldosteronism

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Introduction

In Australia, one's 5-year risk of developing cardiovascular disease (CVD) is predicted using the National Vascular Disease Prevention Alliance (NVDPA) CVD risk algorithm. However, the risk calculator does not consider Primary Aldosteronism (PA) – a treatable syndrome accounting for 5-10% of hypertension in primary care – despite PA conferring a higher risk of coronary artery disease, atrial fibrillation and stroke than blood pressure (BP)-matched Essential Hypertension (EH). We hypothesise that the NVDPA algorithm fails to capture the additional CVD risk associated with PA.

Methods

The NVDPA CVD risk algorithm was retrospectively applied to patients with sufficient data attending an Endocrine Hypertension Service over 4 years. CVD risk scores of PA and EH patients were compared using Chi-square tests and multivariable logistic regression.

Results

Of 109 patients (66 PA, 43 EH; mean age 54 years; 44% female), those with PA had higher systolic BP than patients with EH (median 148 vs 138mmHg, p=0.021). Calculated 5-year CVD risk was low (<10%) in 70% (n=46) of patients with PA and 79% (n=34) of those with EH, and moderate-to-high in 30% (n=20) of patients with PA and 21% (n=9) of those with EH (p=0.279). After accounting for systolic BP, having PA had no significant association with moderate-to-high CVD risk classification (aOR 1.45, 95% CI 0.29, 8.65).

Discussion

In our cohort, the majority of patients with PA were classified as having low 5-year CVD risk. After accounting for differences in BP, they were not more likely to be classified as having moderate-to-high CVD risk compared to those with EH, despite their worse BP-matched prognosis as identified in recent literature.

Conclusion

The NVDPA risk algorithm underestimates the true risk of developing CVD in patients with PA, which may lead to suboptimal treatment and impaired outcomes.

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Selpercatinib treatment of *RET* mutated cancers is associated with gastrointesetinal adverse effects on radiology and histology

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Background: Identifying molecular alterations in thyroid cancer has led to successful targeted therapies particularly in *RET* (REarranged during Transfection) altered cancers. Medullary and differentiated thyroid cancer can harbor RET alterations upregulating intracellular oncogenic pathways, including RAS/RAF/ERK and P13-kinase/AKT pathways. In Phase II studies, selpercatinib, a novel selective RET inhibitor has shown objective responses of >70% and its appeal lies in its efficacy, but also its tolerable adverse effect profile compared with other MKIs. Gastrointestinal SE have not yet been reported in these patients.

Methods: Clinical data from 20 patients enrolled in a selpercatinib clinical trial at Royal North Shore Hospital were retrospectively reviewed. CT scans as per protocol were analysed by radiologists for signs of oedema and scored for inflammation.

Results: 10/20 (50%) of patients reported gastrointestinal adverse effects. The most common symptoms were changes in bowel habits (n=8), abdominal swelling (n-7), abdominal discomfort (n=7), anorexia and nausea. Weight changes were also observed and dose reductions occured in 40% of patients. Endoscopy and biospies were performed in 4 patients. Histopathology demonstrated mild non-specific mucosal oedema characterised by myxoid change in the lamina propria of the stomach and subtle accumulation of oedema fluid in the tips of the duodenal villi and submucosa. There was no histological evidence of inflammation and no increase in intra-epithelial lymphocytes.

Conclusion: We describe the clinical and histopathological presentation of a novel adverse effect of selepercatinib seen in a significant proportion of patients. This previously unreported adverse effect necessitated dose reductions. CT changes can be correlated with onset of symptoms. The presence of mucosal oedema observed histologically in conjunction with the radiological findings of congestion suggest that bowel wall oedema may be a mechanism of abdominal pain in these patients.

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Venous thromboembolism in transgender individuals: a case series

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Background: Hormone therapy is an effective and important part of gender affirming care for many trans and gender diverse (TGD) individuals. In cisgender women, exogenous oestrogen carries a well-recognised dose-dependent thrombotic risk, heightened in the presence of other venous thromboembolism (VTE) risk factors such as inherited thrombophilia and obesity. The prothrombotic risk of testosterone replacement is less well established. The prevalence of VTE during hormone therapy, and changes in risk over time, remain unknown in TGD individuals.

Case Series: VTE in six TGD individuals is presented with clinical and management features summarised in the table. Patients 1 to 4 developed VTE during gender affirming hormone therapy. Patient 1 developed lower limb deep vein thrombosis (DVT) after one month of transdermal oestradiol, whereas Patients 2 to 4 developed VTE after 1-19 years on oral oestrogen. Patient 2 developed DVT in the context of oestradiol dose increasing from 4 to 5 mg/day due to low serum oestradiol (187 pmol/L). Patient 3 had previous provoked DVT following orchidectomy. Patient 4 was lost to follow-up prior to VTE presentation. Patient 5 was on intramuscular testosterone for Klinefelter syndrome when they developed VTE and later commenced oestrogen as part of gender transition. All patients were anticoagulated: Patient 1 with warfarin and others with direct oral anticoagulants. Patient 2 discontinued hormonal therapy due to concerns regarding alopecia from her anticoagulation. Patient 5 ceased testosterone and commenced oestrogen as part of her transition.

Conclusion: Hormone therapy carries a risk of VTE in TGD individuals. Clinical and mechanistic studies are needed to further our understanding of VTE risk and factors which may guide VTE risk stratification and management. Further research is required to inform optimal management of hormone therapy and anticoagulation in TGD individuals with and without a prior history of, or risk factors for, VTE.

	Age (years) at first VTE	Hormone therapy at time of VTE (other than testosterone, doses listed are daily doses)	Duration on hormone therapy prior to VTE	Hormone levels at time of VTE (if available)	VTE	VTE management	Post-VTE hormone therapy
		Individuals on gender-affirming hormone therapy at time of first VTE					
1	31	E2 (transdermal; 1mg gel) + CPA 25 mg	1 month	E2: 236 pmol/L T: 0.7 nmol/L	DVT	Warfarin	E2 (transdermal) + CPA
2	38	E2 (oral; 5 mg) + CPA 12.5 mg	14 years (but E2 dose increased 1 month prior - 4mg to 5mg)	E2: 187 pmol/L T: 1.2 nmol/L	DVT and PE	DOAC	Nil
3	34	1. Ethinylestradiol (oral; MG50) 2. E2 (transdermal; 1 mg gel and oral; 2 mg)	1. 1 year 2. 11 years	E2: 440 pmol/L T: 0.4 nmol/L	1. DVT 2. DVT	DOAC	E2 (transdermal)
4	59	E2 (oral; 2mg)	19 years		Bilateral PE	DOAC	E2 (transdermal)
		Individuals who commenced gender-affirming hormone therapy after VTE					
5	53	Testosterone undecanoate (intramuscular; for Klinefelter syndrome); Heterozygous Factor V Leiden mutation	>15 years	T: 14.3 nmol/L Haematocrit 0.46	DVT and PE	DOAC	Ceased T; E2 (transdermal) commenced 1 year after DVT/PE
6	33	Nil; Homozygous Factor V Leiden mutation	N/A		DVT x 2	DOAC	E2 (transdermal) + CPA commenced 2 years after DVTs

Table: Summary of cases. VTE, venous thromboembolism. E2, oestradiol. CPA, cyproterone acetate. T, testosterone. DVT, deep vein thrombosis. PE, pulmonary embolism. DOAC, direct oral anticoagulant. MG50, microgynon-50 (ethinylestradiol + levonorgestrel).

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An audit of inpatient endocrinology admissions and consultations at a metropolitan hospital

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Background and Aim:

The composition of non-diabetes endocrine admissions and referrals for consultation to the Endocrine Service is not well understood and we aimed to characterize these occasions of service at a metropolitan hospital.

Methods:

Consecutive endocrinology admissions and consultation data were collected by two Endocrine Advanced Trainees between February 2020 and January 2021. Standard demographic data, primary +/- secondary endocrine issue and referring team details were collected for all encounters. Diabetes as a primary referral issue was excluded.

Results:

428 of 1454 total encounters were related to non-diabetes endocrine issues (29.4%), with 83.9% (n=359) consultation referrals and 16.1% (n=69) admissions under our service. 63.3% (n=271) were female (61.9±19.8 years) and 36.7% (n=157) were male (61.6±17.3 years). The respiratory (n=57), cardiology (n=56) and geriatrics (n=53) teams provided the most number of non-diabetes endocrine referrals and represent 38.5%, 28.3% and 34.4% of their total endocrine consult load respectively. Electrolyte disorders formed the majority of encounters, comprised of sodium (n=99), calcium (n=75) and potassium disorders (n=8). Thyroid referrals were next common, including thyroid function disorders (n=98), thyroid mass (n=16), pregnancy thyroid disorders (n=9)

and thyroid cancer (n=2). The remaining referral categories include adrenal disorders (n=42), pituitary disorders (n=18), other (n=14), osteoporosis/ bone disorders (n=12), non-diabetic hypoglycaemia (n=10), obesity (n=10), lipid disorders (n=7), hypertension (n=5), neuroendocrine tumour (n=2) and gonadal disorders (n=1). 18 patients had diabetes as a secondary referral issue. Geriatrics provided 21/75 of calcium disorders whereas cardiology provided 28/121 of thyroid disorders, reflective of the underlying pathologies seen in these specialties. The most common admission diagnosis was sodium disorders, specifically hyponatraemia (36/69 admissions), adrenal insufficiency (7/69) and other (7/69).

Conclusion:

Endocrinology is a busy clinical service in our hospital and a significant proportion of the workload is non-diabetes endocrinology. Service audits provide meaningful information on the distribution and patterns of referrals.

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Audit of selective arterial calcium stimulation with right versus bilateral hepatic venous sampling: a single-site experience

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Background and Aim

In patients with endogenous hyperinsulinaemic hypoglycaemia, negative oral sulphonylurea screen, negative insulin antibodies, and inconclusive imaging, selective arterial calcium stimulation (SACST) with hepatic venous sampling is the next step in insulinoma localisation[1-3]. The Harmonisation Protocol advises sampling from the right hepatic vein only, however our local preference has been bilateral venous sampling. This audit assesses the concordance rate between the two hepatic veins in SACST[2].

Methods

At Westmead Hospital, six patients with seven SACSTs were retrospectively identified between 2002 and 2021, all with bilateral hepatic venous sampling. Protocols differed slightly in terms of collection timepoints, stimulated arteries and the second hepatic vein sampled (left vs. middle). A relative-fold increase in hepatic venous insulin concentration (rHVI) of ≥2 at any timepoint compared to baseline is positive.

Results

6/7 SACSTs yielded positive results in ≥1 artery, indicating good selection of subjects. 20 out of 37 stimulated arteries were positive (54.1%). Allowing for minor variations in low concentrations of both basal and stimulated insulin concentrations, 33/37 (89.2%) stimulations produced concordant results between the hepatic veins sampled. The 4 discordant stimulations occurred in 2 patients with discordant results at several different time points. One patient exhibited vastly different baseline and stimulated insulin levels between the hepatic veins, and the other had widely fluctuating insulin levels and several elevated baseline insulin levels, suggestive of either a sustained response to the previous stimulation or insufficient time between sample collection.

Conclusion

This audit demonstrates the need to ensure accurate sample collection and adequate timing between subsequent arterial stimulation. After excluding samples with likely false discordance due to minor variations in insulin concentrations, the concordance rate between the hepatic veins is sufficient to support sampling from the right hepatic vein only during SACST, which may reduce the potential for collection errors.

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Effect of bariatric surgery on serum triglyceride levels in obese Sri Lankan adults: A follow up of 18 months

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Introduction

Bariatric surgery is the intervention of choice for weight loss in morbid obesity. Bariatric surgery results in significant improvement in all components of metabolic syndrome. Elevated serum triglycerides are an independent risk for cardiac disease. Some studies suggest that reduction of serum triglycerides result in reduced cardiac risk.

Objective

To assess the effect of bariatric surgery on serum triglyceride levels in Sri Lankan adults.

Methods

Pre-operative and follow up weight loss data and serum triglyceride levels of patients who underwent bariatric surgery at the Colombo South Teaching Hospital were assessed. Out of 240 patients, 120 patients with intact data were selected for analysis. The significance of triglyceride level change was assessed by paired sample t test.

Results

Overall 76.2% were females. Mean age (SD) was 37.8 (10.6) years. Mean pre-operative body weight and body mass index were 113.6 (23.6) kg, and 45.0 (6.8) kg/m^2 respectively. The mean pre-operative serum triglyceride level was 135.0 mg/dl (56.1). The mean percentage body weight loss at 1, 3, 6, 9, 12 and 18 months post-operatively were 9.6% (3.9) kg, 16.7% (4.5) kg, 23.2% (5.9), 26.7% (6.8), 28.4% (7.4) and 28.8% (8.7) respectively. The mean serum triglyceride reduction at 1, 3, 6, 9, 12 and 18 months post-operatively were 22.9 (47.9) mg/dl, 18.0 (49.6) mg/dl, 32.6 (52.2) mg/dl, 37.2 (56.4), 41.9 (44.7) and 44.5 (54.1) respectively ($p<0.001$).

Conclusion

Bariatric surgery results in a significant reduction of serum triglyceride levels as early as 1 month after surgery. This effect seems to progressively increase till 9-12 months with plateauing afterwards. The possible cardiac risk reduction gained by reduction of serum triglycerides need to be considered when patients are being considered for bariatric surgery.

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A Retrospective Review of Short Synacthen Test Requesting Patterns in Queensland Public Hospitals

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The Short Synacthen Test (SST) is a widely used investigation of adrenocortical function and is considered the gold standard for diagnosing primary adrenal insufficiency (PAI) by the Endocrine Society[1]. The standard high-dose test involves administration of a supraphysiological dose (250mcg) of synthetic adrenocorticotropic hormone (ACTH), followed by serial measurement of plasma cortisol. The SST protocol can be time consuming and labour intensive to perform and not without risk, with anaphylaxis to Synacthen previously reported[2].

With the development of the modern ACTH assay, guidelines for investigating PAI have shifted to recommend paired measurement of serum cortisol and plasma ACTH initially, with progression to SST only in equivocal cases[1]. Other indications include evaluation of adrenal suppression after exogenous steroid use, suspected 21-hydroxylase deficiency and other causes of adrenal hyperplasia.

The SST remains frequently requested in public hospitals within Queensland and is also one of the only dynamic tests of endocrine function requested by non-endocrinologists. Our clinical experience suggests that the SST is not always performed for a generally accepted indication or in the appropriate clinical setting.

Our retrospective audit therefore seeks to evaluate SST requesting patterns across public hospitals in Queensland, using data collected by Pathology Queensland between January 2020 to January 2021. The electronic medical record of these patients will be accessed to determine the indication for the test, as well as which specialty requested the test, and whether there were documented SST-related adverse events. Our primary objective is to determine the reasons for performing SSTs within Queensland public hospitals and to compare this with existing recommendations.

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Challenges in Oncofertility – Manipulation of the hypothalamic-pituitary-ovarian axis to provide an opportunity for future pregnancies

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Oncofertility is an expanding subspecialty that bridges oncology, reproductive endocrinology and assisted reproductive technology to improve the options for the reproductive future of cancer survivors. Oncofertility is becoming the standard of care

in prepubertal, adolescent and young adult cancer patients who are at risk of infertility due to their cancer or gonadotoxic treatment. Westmead Fertility Centre has been providing oncofertility services since 1989. We describe two cases that highlight the challenges encountered in oncofertility.

The first case is an 18-year-old female with newly diagnosed Hodgkin's lymphoma. She initially presented with spinal cord compression requiring immediate surgical decompression. Urgent chemotherapy was indicated due to high tumour burden. The challenge in this case is ensuring patient safety whilst preserving fertility with minimal delay to cancer treatment. The patient underwent ovarian stimulation with Gonadotrophin-releasing hormone (GnRH) antagonist protocol and oocyte collection under local anaesthesia without complications.

The second case is a 39-year-old female with newly diagnosed triple positive breast cancer. She has a long-term partner and has no children. The challenge in this case is to minimize estrogen exposure on estrogen receptor positive breast cancer during ovarian stimulation. The patient underwent ovarian stimulation with letrozole cover which effectively attenuated the estrogen level to that of an unstimulated cycle.

In both cases, fertility preservation was achieved without compromising patient safety and cancer treatment owing to the advances in assisted reproductive technology. The knowledge in reproductive physiology enabled the manipulation of the hypothalamic-pituitary-ovarian axis to achieve multiple follicular recruitment through ovarian stimulation. GnRH antagonists are used for pituitary suppression to prevent premature ovulation and to override dominant follicle selection whilst exogenous gonadotrophins are used to enable the maturation of multiple follicles. Ovarian stimulation has become an integral part in fertility preservation for female cancer patients with impending gonadotoxic treatment.

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Lithium induced silent thyroiditis

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A 30-year-old man, who has been taking lithium carbonate 1350 SR daily for 2 years for management of treatment refractory schizoaffective disorder was admitted into the mental health ward for a relapse of schizophrenia symptoms. He had recent increase in the dose of Lithium due to low level. He had no symptoms suggestive of thyrotoxicosis Neither any features suggestive of thyroiditis like neck pain, fever, or any preceding viral illness. On routine testing of thyroid function, he was found to be thyrotoxic with fT4 25.3 pmol/L (9.0-25) pmol/L, fT3 11.2 pmol/L (3.5-6.5) pmol/L, TSH 0.01 mIU/L (0.40-4.00) mIU/L. His lithium level was 0.7 milliequivalents per liter (therapeutic range 0.4-1.2). Anti-thyroid peroxidase antibody 36 U/ml (Reference range <60), anti-thyroglobulin antibody U/ml <15, thyroid stimulating hormone immunoglobulin (TSI) <0.10 IU/L. A technetium thyroid uptake scan demonstrated absent uptake as shown in figure 2. Ultrasound of the thyroid gland showed a normal sized thyroid gland with no nodules, normal vasculature, and homogenous echotexture. At this stage, a diagnosis of lithium-induced silent thyroiditis was the working diagnosis. Further investigations excluded autoimmune thyroid disease. With medical treatment and cessation of lithium, His thyroid function improved.

Treatment:

He was initially started on carbimazole 5 mg twice per day and the dose was subsequently doubled and prednisolone 25 mg per day was added. Carbimazole reached a maximum dose of 20 mg twice per day. Due to poor initial response to medical treatment and ongoing high T4 and T3 , a Thyroidectomy was considered but was not needed due to response to medical treatment as seen in his last thyroid function test showed a TSH of 0.04 mIU/L, a fT4 of 14.2 pmol/L and a fT3 of 5.6 pmol/L. His carbimazole dose was slowly reduced and he is awaiting a follow up with the endocrinology team

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Immune checkpoint inhibitor induced diabetes mellitus with Pembrolizumab

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An 81-year old female with a background of metastatic melanoma on pembrolizumab with no prior history of diabetes was brought in to the emergency department with polyuria, polydipsia and weight loss. The initial assessment was consistent with severe diabetic ketoacidosis (DKA) and prerenal acute kidney injury with no clinical evidence of infection. The patient was treated with fluid resuscitation and an insulin infusion and eventually transitioned to a basal-bolus insulin regime which was continued after discharge.

Diabetes autoantibody screen returned negative, and she was diagnosed with immune checkpoint inhibitor induced diabetes mellitus (ICI-induced DM) due to pembrolizumab. The patient has clinically improved and pembrolizumab was continued.

The aim of this report is to highlight the importance of recognising ICI-induced DM as a rare immune-related adverse event (irAE) seen in patients receiving PD-1/PD-L1 inhibitor therapy and provide clinicians with insight into immune checkpoint endocrinopathies with an emphasis on diabetes and DKA.

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The negative impacts of depot medroxyprogesterone acetate on perimenopausal symptoms and bone mineral density in mid-life

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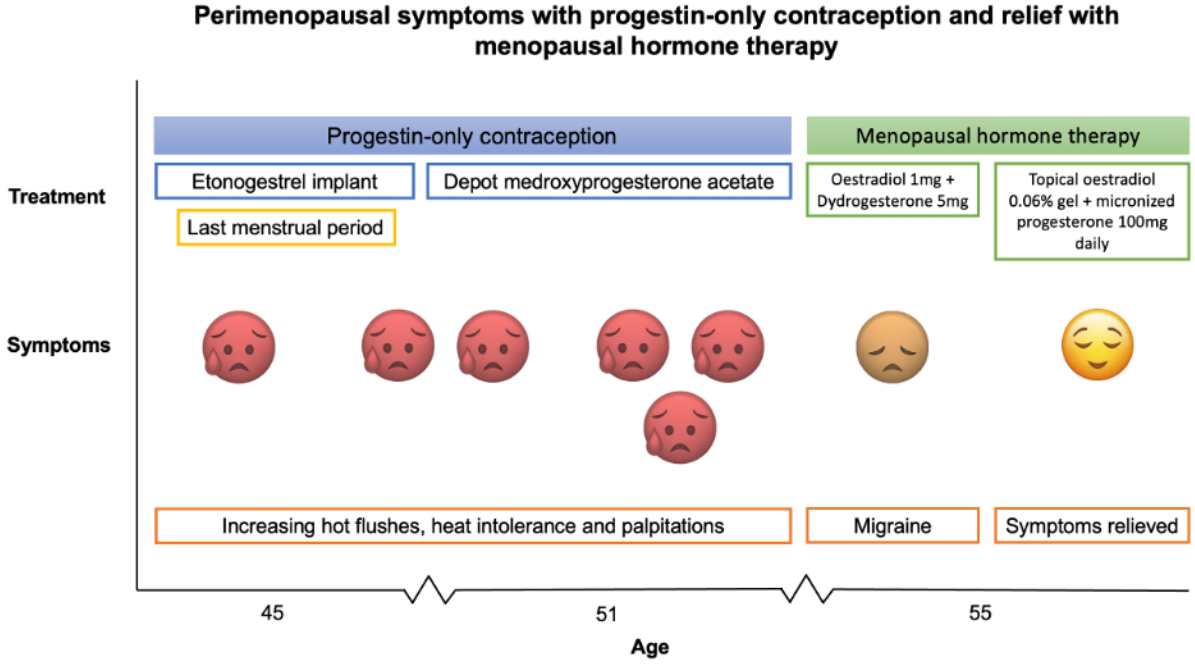
A 55-year-old woman presented for endocrinologist assessment following 12 years of episodic sweats, heat intolerance, palpitations and migraines. She had undergone extensive investigations for these symptoms without a cause identified, but was recently diagnosed with tachycardia associated cardiomyopathy. The only other past history included a coccyx fracture 6 years ago. Physical examination revealed an overweight BMI (29kg/m^2) without any features of endocrinopathy.

On further history her last menstrual period occurred at age 45 coinciding with vasomotor symptoms onset and insertion of an etonogestrel implant. This continued to be utilised until 51 years of age when she was changed to intramuscular depot medroxyprogesterone acetate (DMPA) three monthly, for two years during which her symptoms intensified.

Further investigation identified a low oestradiol level and elevated gonadotrophins, whilst DXA revealed osteoporosis (lumbar spine T score -3.3). On assessment, her symptoms were attributable to peri-/postmenopausal status.

Menopausal hormone therapy (MHT), oral 1mg oestradiol/ 5mg dydrogesterone was therefore commenced but soon ceased due to migraine with minimal improvement in vasomotor symptoms. She was then switched to topical oestradiol 0.06% gel and micronized progesterone, 100mg nocte. Her vasomotor symptoms, palpitations and general heat intolerance subsequently resolved at two-month review without worsening migraines.

This case illustrates the hypoestrogenic effect of progestin-only contraception which has overlapped the menopause transition, both contributing to osteoporosis. Progestin-only contraception use over age 50 causes reduction in bone mineral density and elevates cardiometabolic risk (1,2). DMPA, in particular, is associated with these adverse effects and thus not recommended as first line contraception in women over 45 and should be discontinued over 50. A literature review of such adverse effects attributable to progestin only contraception will be provided. In conclusion, perimenopausal symptom recognition with MHT consideration is the key to improving quality of life and avoiding adverse health outcomes.



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When tissue is the issue: A case of adrenocortical cancer, neurofibromatosis type 1 and gender affirming hormone therapy

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Background: Adrenocortical cancer (ACC) is a rare malignancy which occurs more frequently in females.[1] Neurofibromatosis type 1 (NF1) has a clear link with a number of endocrine tumours, including reports of nine patients with ACC.[2] Normal adrenal glands express oestrogen and progesterone receptors but the role of reproductive hormones on ACC tumorigenesis is unclear. While the development of benign and malignant tumours with gender-affirming hormone therapy (GAHT) in male-to-female (MtF) transgender individuals has been described[3], the effect of GAHT on NF1 and ACC remains unknown.

Case: We present the case of JS, who was diagnosed with NF1 at birth. At age 10, CT abdomen to investigate a hydrocele revealed an incidental 13cm right-sided retroperitoneal mass. A paravertebral paraganglioma was suspected but no excess catecholamine secretion was demonstrated. Post-resection, histopathology confirmed extra-adrenal ACC, based on high Ki67 of 50%, vascular invasion and ultrastructural features consistent with steroid-secreting cells. CT/FDG-PET demonstrated no primary adrenal or metastatic lesions. The following year, surveillance imaging revealed a 30mm right adrenal mass and 15mm left lower lobe lung lesion, treated with right adrenalectomy, pulmonary lesion resection and adjuvant chemotherapy. Surveillance CT the next year showed an 8mm left upper lobe lung lesion, treated with resection and adjuvant mitotane. There was no further recurrence. At age 23, JS disclosed gender dysphoria and a desire for MtF transition. Archived adrenal tissue was assessed for oestrogen receptor expression and showed focal areas of moderate immunopositivity in <10% of tumour cells. After discussion of the potential risks of cancer recurrence and benefits of GAHT, JS opted to commence GAHT.

Conclusion: This is the tenth report raising a possible link between NF1 and ACC. Recognition of individuals on GAHT with higher baseline tumour or malignancy risk is crucial and clinicians should consider increased frequency of cancer screening in these individuals.

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An under-recognised association: hypocalcaemia and intravenous iron polymaltose

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We present two cases of hypocalcaemia post intravenous iron polymaltose infusion. Hypophosphataemia is a well-recognised phenomenon following intravenous iron,[1,2] however hypocalcaemia has been rarely documented.[3,4]

Case 1 is a 62-year-old female who presented with symptomatic hypocalcaemia ten days after intravenous iron polymaltose infusion with a nadir calcium of 1.80mmol/L. Hypophosphataemia preceded hypocalcaemia by five days. She was vitamin D deficient; parathyroid hormone (PTH) and FGF-23 were significantly elevated; 1,25-dihydroxyvitamin D was low. Cholecalciferol and calcitriol (0.5mcg twice daily) were commenced at presentation and calcitriol was subsequently weaned over four weeks. Two months after calcitriol cessation, calcium and phosphate had normalised and PTH had significantly reduced (see Table 1).

Case 2 is a 59-year-old female who developed symptomatic hypocalcaemia seven days after intravenous iron polymaltose with a nadir calcium of 1.82mmol/L. She had a background of glucocorticoid-induced osteoporosis treated with denosumab with the last dose four months prior to presentation, as well as vitamin D deficiency treated with high dose oral cholecalciferol. Vitamin D level was normal and PTH and FGF-23 were significantly elevated (see Table 1). Calcitriol 0.75mcg daily was commenced and subsequently weaned. Calcium normalised three months later.

Parenteral iron is complexed with carbohydrate moieties including carboxymaltose and polymaltose, which prevent the degradation of FGF-23.[5,6] Hypophosphataemia therefore develops due to excess FGF-23 inhibiting phosphate reabsorption in the renal tubules.[7] FGF-23 also inhibits calcitriol production which reduces intestinal absorption of phosphate and calcium, thus exacerbating hypophosphataemia and potentially causing hypocalcaemia.[7]

We hypothesise that hypocalcaemia develops primarily in patients with additional risk factors such as vitamin D deficiency or denosumab exposure, as described above. We are conducting a retrospective audit to assess the frequency of hypocalcaemia post intravenous iron infusion, as this complication may be under-recognised. Meanwhile, these cases highlight the need for clinicians to be cognisant of this association.

Table 1. Biochemistry at presentation and on 3 month follow up.				
	Case 1		Case 2	
	Presentation	3 months later	Presentation	3 months later
Corrected calcium (2.10-2.60mmol/L)	1.80 (↓)	2.28 (n)	1.82 (↓)	2.38 (n)
Ionised calcium (1.15-1.35mmol/L)	1.06 (↓)	1.18 (n)	1.04 (↓)	1.23 (n)
Phosphate (0.75-1.50mmol/L)	0.34 (↓)	1.04 (n)	0.59 (↓)	1.62 (n)
Magnesium (0.70-1.10mmol/L)	0.72 (n)	0.79 (n)	0.83 (n)	0.72 (n)
Parathyroid hormone (1-7pmol/L)	35 (↑)	11 (↑)	47 (↑)	5.7 (n)
Vitamin D (50-150nmol/L)	36 (↓)	62 (n)	81 (n)	37 (↓)
1,25-dihydroxy vitamin D (48-190pmol/L)	36 (↓)	124 (n)	-	-
FGF-23 (23-95ng/L)	414 (↑)	Not repeated	591 (↑)	Not repeated

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An unusual growth in the thyroid

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A 69-year-old female with a background of Hashimoto's thyroiditis presented to endocrinology clinic with a rapidly enlarging neck mass. She had no B symptoms of malignancy. On examination she had an unusually firm goitre. Ultrasound and computed tomography demonstrated an enlarged thyroid with abnormal diffuse spongiform appearance without discrete nodules. Thyroid lymphoma was suspected clinically and FNA with flow cytometry and subsequent core biopsy confirmed the diagnosis of high-grade B-cell lymphoma. Bone marrow aspirate and trephine (BMAT) was negative, and fluorodeoxyglucose-positron emission tomography (FDG-PET) excluded advanced disease (see Figure 1a). Treatment with four cycles of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone followed by adjuvant radiotherapy resulted in complete metabolic response. Figure 1b demonstrates the post-treatment FDG-PET which reveals minor residual thyroid uptake, likely related to underlying Hashimoto's disease.

Primary thyroid lymphoma has an incidence of 2 cases per million persons and accounts for 2% of extra-nodal lymphomas and <5% of thyroid malignancies.[1,2] It is more common in females and has a median age of diagnosis of 65 years.[2] The only established risk factor is Hashimoto's thyroiditis which confers a 60-fold increased risk.[1] The majority of primary thyroid lymphomas are diffuse large B-cell lymphomas.[1,3] Diagnosis is made with core biopsy and staging requires BMAT and FDG-PET.[2,3] 80% of patients have limited disease with 5-year overall survival of >80%.[2] Treatment is typically with chemotherapy and radiotherapy which improves survival compared with chemotherapy alone.[2] The addition of rituximab also significantly improves survival.[2,4] Surgery is seldom required and does not affect outcomes.[2,5] Even patients with severe obstructive symptoms rarely require surgery as prednisolone rapidly improves such complications, including airway compromise.[2,3]

Primary thyroid lymphoma is an important diagnosis to consider as rapid growth can result in significant compressive complications. Prognosis is favourable but treatment is distinctly different from the usual management of thyroid malignancy.

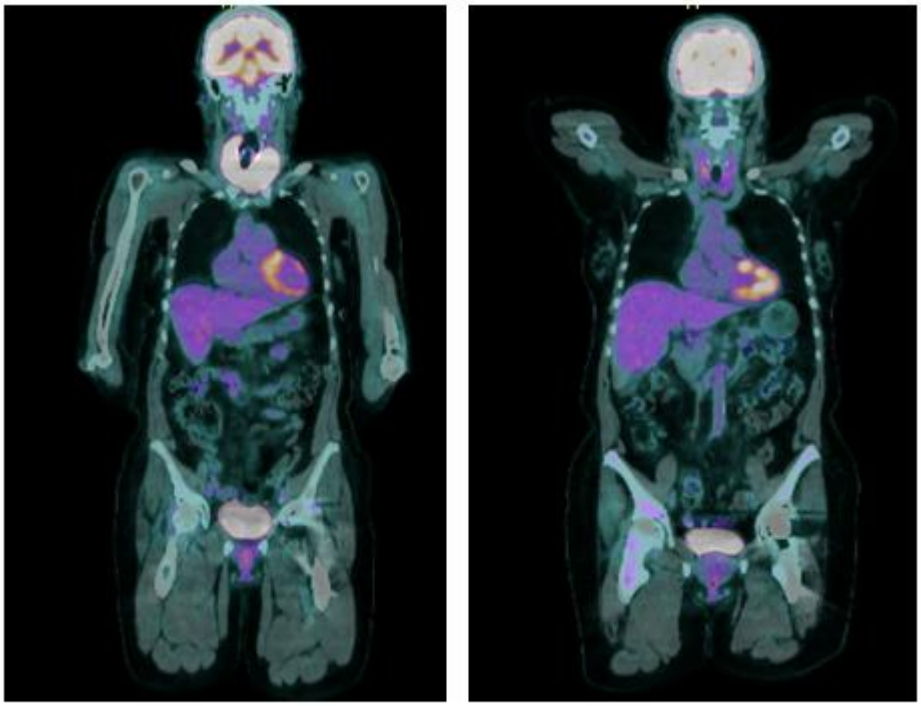

Figure 1. Figure 1a on the left demonstrates the initial FDG-PET at diagnosis which shows thyroid uptake and no nodal or other extra-nodal disease. Figure 1b on the right demonstrates the FDG-PET after treatment with significantly reduced FDG uptake in the thyroid and no new sites of lymphoma.

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An adrenal dilemma : to resect or not to resect ?

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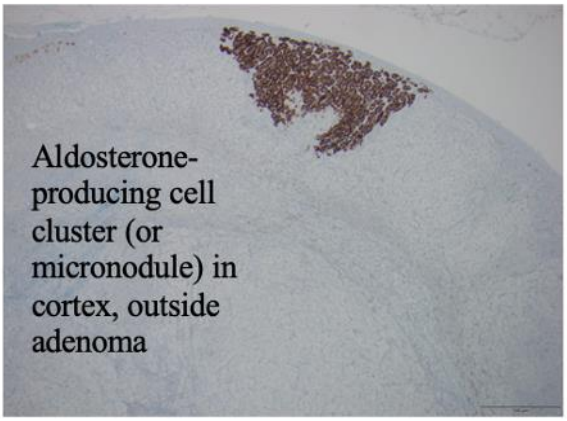
A 39-year-old male was referred for evaluation of a 8 x 7.3 x 7.6cm left adrenal cystic mass in the context of hypertension. He was diagnosed with hypertension at age 33 years and experienced poor control despite four antihypertensive medications (perindopril 10mg daily, lercanidipine 20mg daily, moxonidine 400mcg daily and prazosin 0.5mg daily). He also had hypokalaemia, obstructive sleep apnoea and obesity. On initial assessment, his blood pressure (BP) was 175/100mmHg. There were no signs of cortisol excess. His initial biochemistry showed a potassium level of 3.2mmol/L with normal renal function. To assess the functionality of the left adrenal cystic mass, he had a 1mg dexamethasone suppression test, 24-hour urine free cortisol measurement and plasma metanephrines which were normal. To facilitate testing for aldosterone excess, perindopril and lercanidipine were changed to hydralazine 50mg BD and verapamil SR 180mg daily while prazosin and moxonidine were continued. Supplementation with 2400mg of potassium chloride per day was required to achieve normokalaemia. Plasma aldosterone-renin-ratio (ARR) was measured twice: the first was 88 (aldosterone 1140pmol/L, renin 13mIU/L) while the second was 268 (aldosterone 1070pmol/L, renin 4mIU/L) with a serum potassium of 3.6mmol/L and eGFR >90mL/min. Saline

suppression test was bypassed. He underwent adrenal vein sampling (AVS) which demonstrated clear-cut right-sided lateralization with a lateralization index of 8.6 and contralateral suppression prior to ACTH stimulation. However, the right-sided lateralization was blunted following ACTH stimulation with lateralization index falling to 2.6 and loss of contralateral suppression. A right adrenalectomy was performed. Histopathology showed a 10mm cortical adenoma. Immuno-histochemistry showed absence of CYP11B2 staining within the adenoma but presence of aldosterone-producing cell clusters outside the adenoma (Figure 1). Four months post-surgery, he only required two antihypertensive medications to control his BP at <140/90mmHg, however biochemical cure is yet to be achieved (ARR 165, aldosterone 610pmol/L, renin 3.7mU/L).

Figure 1. The patient underwent unilateral right sided laparoscopic adrenalectomy and histopathology showed a 10mm cortical adenoma with absence of CYP11B2 staining but showed aldosterone-producing cell clusters outside the adenoma.



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A Tale of Two Adenomas: A Case of Autonomous Cortisol Secretion

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A 37 year-old lady was found to have bilateral adrenal incidentalomas. Clinically, she was obese (BMI 36), normotensive, and with no cushingoid features. CT Abdomen showed bilateral hypodense adrenal lesions, measuring 2.1 x 1.4 cm on the right and 2.6 x 1.7 cm on the left. Initial investigation showed no evidence of hormonal hypersecretion, except for a suppressed ACTH.

The patient was under regular clinical, biochemical and radiological surveillance for the next ten years. She developed significant weight gain, hypertension, and osteopenia of the spine with Z-score -1.9 at L3-L4 (pre-menopausal). Repeat testing showed abnormal cortisol post 1 mg dexamethasone suppression test of 101 nmol/L, suppressed ACTH and DHEAS but no overt Cushing's syndrome. Midnight salivary cortisol and 24 hour urinary free cortisol measurements were normal.

Both adrenal adenomas had increased by 30% and 50% from baseline (Figure 1). Norcholesterol scan confirmed hyperfunctioning of the left adrenal lesion for which she underwent left adrenalectomy. Histology showed nodular adrenocortical hyperplasia. Post-operatively, she successfully lost 20 kg in 18 months, with improvement in lipid profile, blood pressure and bone density at the spine. ACTH and DHEAS, which were previously suppressed, normalised.

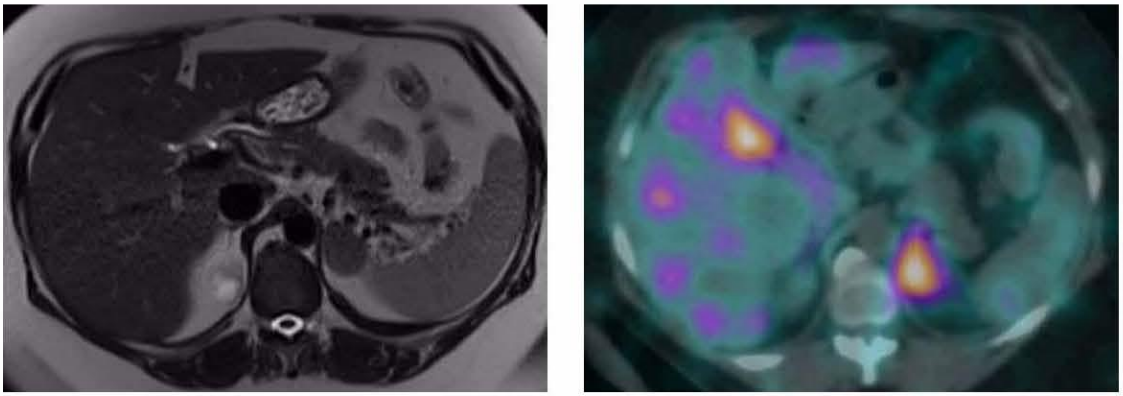

Figure 1a. MRI Abdomen (left) demonstrating right adrenal adenoma 2.8 x 2.4 cm and left adrenal adenoma 3.4 x 2.7 cm; and ***1b.*** Norcholesterol scan (right) demonstrating hyperfunctioning of left adrenal adenoma.

Autonomous cortisol secretion (ACS) is characterised by ACTH-independent cortisol overproduction from adrenal incidentalomas without clinical features of overt Cushing's syndrome. It is associated with metabolic consequences of obesity, hyperlipidaemia, hypertension, osteoporosis and increased cardiovascular mortality. ACS is more common in bilateral adrenal adenomas compared to unilateral lesions. Diagnosis and management are challenging due to controversies and discrepancies in current clinical guidelines and lack of long-term outcome data. Individualised clinical and risk assessment is important particularly in those with significant change in clinical features. The use of the 1 mg dexamethasone suppression test demonstrates the highest sensitivity for screening for ACS, with low/suppressed ACTH and DHEAS as useful adjuncts in the diagnosis.

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Dilated cardiomyopathy associated with thyroid storm due to Graves' disease

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Background: Dilated cardiomyopathy (DCM) is an uncommon manifestation of hyperthyroidism. We describe a case of DCM associated with Graves' disease.

Case: A 60-year-old man presented with acute dyspnoea and palpitations. Pertinent examination findings included mild disorientation, temperature of 37.9ºC, hand tremors, a large goitre, thyroid bruit and lid lag. Heart rate was irregularly irregular at 240 beats/minute, indicative of atrial fibrillation, with blood pressure of 120/90mmHg and signs of mild congestive cardiac failure. Biochemistry revealed freeT4 of >100pmol/L(range:12-22), freeT3 of 42.7pmol/L(range:3.0-7.8) and undetectable thyroid-stimulating hormone (TSH). Of note, pathology undertaken two years ago following an emergency department presentation with fatigue demonstrated severe hyperthyroidism, but this result was inadvertently missed, and he had not sought any medical care until this presentation. He was diagnosed with thyroid storm complicated by heart failure. Propylthiouracil 200mg 6-hourly and intravenous hydrocortisone 100mg 6-hourly were initiated. Oral metoprolol and intravenous esmolol were administered for rate control but unfortunately led to cardiogenic shock. Cardioversion resulted in sinus rhythm and a transient blood pressure improvement. Inotropes were subsequently commenced. Echocardiogram revealed left ventricular (LV) systolic dysfunction with septal wall hypokinesis, left atrial enlargement and increased LV filling pressure, consistent with DCM. He was weaned off inotropes after 48 hours. TSH-receptor antibodies returned positive, confirming Graves' disease. Ten days later, improved thyroid function (freeT4 19.1pmol/L, freeT3 6.5pmol/L) coincided with normalisation of LV systolic function. He was discharged home

following a 7-week admission. He achieved euthyroidism 3 months later with carbimazole, with no cardiomyopathy recurrence on continued follow-up and was referred for a thyroidectomy.

Conclusion: In contrast to cardiomyopathy of other origins, hyperthyroidism-associated DCM is usually reversible with appropriate treatment. Clinicians should be vigilant of the risk of precipitating cardiac decompensation in patients with hyperthyroidism-induced cardiomyopathy with beta blockers, which are commonly used for rate control and symptomatic relief.

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Diabetes and illicit drug use: severe diabetic ketoacidosis associated with cocaine ingestion

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Introduction: Cocaine use has been reported as a precipitating factor for diabetic ketoacidosis (DKA) in patients with diabetes. The development of DKA in the setting of cocaine use is contributed by the stimulatory effect of cocaine on counter-regulatory hormone release, leading to increased ketoacid production. We report a case of DKA associated with cocaine ingestion.

Case: A 46-year-old man presented with a 1-day history of abdominal pain, nausea and polyuria. He had type 2 diabetes mellitus of 8 years duration and was on metformin monotherapy. There was no personal or family history of autoimmunity. Examination revealed tachycardia and mild epigastric tenderness. Investigations demonstrated: serum glucose 23.6mmol/L (range: 3.5-5.4), pH 7.02 (range: 7.30-7.40) and point-of-care ketone 4.6mmol/L (range: <0.6), consistent with severe diabetic ketoacidosis. Inflammatory markers were raised, likely due to acute inflammatory response to DKA as there was no evidence of an infection. An abdominal CT excluded acute intra-abdominal pathology including pancreatitis. The cause of his DKA was initially unclear and investigations for autoimmune diabetes were under way. Upon further questioning, it was revealed that he had ingested 1 gram of cocaine approximately 16 hours prior to presentation and was hospitalised 2 years ago with DKA associated with cocaine use. Due to the plausible temporal relationship between cocaine use and symptom onset, cocaine ingestion was implicated as the cause of his ketoacidosis. Intravenous insulin infusion was commenced. He was transitioned to subcutaneous insulin following resolution of ketoacidosis. HbA1c was 7.8%. Islet autoantibodies returned negative. He was counselled to avoid cocaine use in the future due to the risk of recurrent DKA.

Conclusion: Cocaine use is a known precipitating factor in the development of DKA. Our case illustrates the importance of considering recreational drug use as a trigger of DKA when the precipitant is not apparent after excluding common causes.

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Lower limb amputations in patients with diabetes-related foot complications in the COVID-19 pandemic

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The COVID-19 pandemic caused disruption to healthcare with concern about individuals delaying or avoiding presentation. In-person reviews have been limited with a major shift towards Telehealth. This has raised concern for the management of diabetes-related foot complications, which has traditionally required in-person visits for debridement, dressings and offloading (1). Delay in access to care can lead to significant mortality, morbidity and loss of limbs (2). Melbourne's first case of COVID-19 was detected in January 2020 and in March 2020 elective surgery was suspended.

Aim

To examine the effect of the COVID-19 pandemic on the rates of lower limb amputations in individuals with diabetes-related foot complications.

Method

This retrospective audit of medical records analysed rates of amputations at a large hospital in Victoria, Australia. Comparisons were made between pre-COVID-19 (January-December 2019) and post-COVID-19 (January-December 2020) onset periods. Descriptive statistical analyses were utilised.

Results

In 2019, 84 lower limb amputations were performed in individuals with diabetes-related foot complications at the Royal Melbourne Hospital (RMH). Of those, 56 occurred in individuals directly admitted under the Diabetic Foot Unit (DFU). In 2020, 96 amputations occurred, 61 in individuals admitted under the DFU. The percentage of individuals admitted under the DFU requiring an amputation was 48% in 2019 and 58% in 2020. Ulcer duration was similar in 2019 and 2020 despite the lockdown and COVID-19 related restrictions. Comparing 2019 and 2020, rates of osteomyelitis were 52% vs. 65%, gangrene 56% vs. 60% and cellulitis 73% vs. 80% (Table 1).

Conclusion

Following the onset of the COVID-19 pandemic, a greater proportion of individuals presented with osteomyelitis, gangrene and cellulitis associated with foot ulcers, which may have contributed to the greater number of amputations performed in 2020. As RMH prioritised in-person reviews during the COVID-19 pandemic, the effect of a shift to virtual healthcare is unknown.

Parameter	2020 group (n=96)	2019 group (n=84)
Age, years	69 +/- 12.8	65 +/- 11.7
Male, n (%)	70 (73)	67 (80)
HbA1c, %	7.8 +/-2.2	8.3 +/- 2.7
PAD, n (%)	84 (88)	70 (83)
Neuropathy, n (%)	92 (96)	79 (94)
Previous ulcers, n (%)	81 (84)	65 (77)
Previous amputations, n (%)	61 (64)	35 (42)
Ulcer duration, weeks	4 (2-12)	4 (2-10.5)
Osteomyelitis, n (%)	62 (65)	44 (52)
Gangrene, n (%)	57 (60)	47 (56)
Cellulitis, n (%)	77 (80)	61 (73)
Intervention, n (%) AKA BKA TMA Minor amputation	 1 (1) 17 (18) 7 (7) 71 (74)	 3 (3.6) 14 (17) 16 (19) 49 (58)

Table 1. Demographics, clinical characteristics and biochemistry. Data are reported as mean +/- SD or median (interquartile range). Minor amputation refers to toe or ray amputation. AKA=above knee amputation; BKA=below knee amputation; TMA=trans-metatarsal amputation.

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Double-Hit lymphoma presenting as acute pituitary failure

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Introduction:

Double-Hit lymphoma (DHL) is a type of high-grade B cell lymphomas with rearrangements of MYC with BCL2 or BCL6 genes. DHL are more aggressive and carry and worst prognosis compared to other Non-Hodgkin lymphomas. Although CNS involvement at diagnosis is frequent and estimated at 10%, isolated involvement of the hypothalamus-pituitary axis is very rare and estimated at a frequency of <0.5% among systemic lymphomas.

Case description:

This case describes a 46 year old female presenting acutely with pituitary failure characterized by headache, fatigue and dizziness. Laboratory test showed severe hyponatremia at 128 mmol/L and acute kidney injury. Emergency CT scan of the head showed abnormal thickening and enhancement of the pituitary infundibulum later confirmed on MRI, with the impression of lymphocytic hypophysitis with a broad differential aetiology. She was prompted started on appropriate hormonal replacement with significant clinical improvement.

Multiple indurate lesions skin over face, shoulders and lower abdomen were ntoed, and preliminary histology suggested likelihood of B cell non-Hodgkins lymphoma. Fluorescence *in situ* hybridisation (FISH) later confirmed rearrangements for MYC and BCL2 genes. Staging CT revealed cardiac and retroperitoneal involvement with obstructive hydronephrosis requiring stenting.

Patient was promptly started on short intensive chemotherapy in view of then performing autologous stem cell transplant. PET scan prior to stem cell transplant confirmed complete remission and patient is regularly monitored while recovering.

Discussion:

Acute panhypopituitarism can be caused by a broad spectrum of aetiologies. Most of them are usually slow to progress, commonly managed in the outpatient settings and often untreatable. This case illustrates an aggressive type of Non-Hodkin lymphoma diagnosed after presenting with pituitary infiltration that responded completely to prompt aggressive chemotherapy.

Severe Hypercalcaemia in Pregnancy and Lactation – Like Mother, Like Baby?

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This case contributes to the limited literature on severe PTH-independent hypercalcaemia during pregnancy and lactation. A 38-year-old primiparous woman presented in early pregnancy with a 6-year history of intermittent mild PTH-independent hypercalcaemia and elevated 1,25-hydroxyvitamin D (1,25VitD) level without identified cause (Figure 1a) or suspicion of calcitriol use.

In early second trimester she developed severe PTH-independent hypercalcaemia (corrected calcium 3.38mmol/L (range 2.1-2.6)) that persisted during her pregnancy (Figure 1b). Her third trimester PTHrp was elevated 42.27pmol/L (non-pregnant range <1.0) with a 1,25VitD (835pmol/L) twice the expected pregnancy range. She had minimal response to intravenous fluids and did not respond to 10-day high-dose glucocorticoid. A modest response to strict low calcium diet (<150g per day) was observed. There was no evidence of end-organ damage until development of pre-eclampsia at 36-weeks. A healthy infant was delivered via emergency caesarean-section at 37-weeks gestation. Maternal hypercalcaemia persisted post-partum and worsened with establishment of lactation. Fluconazole (1-alpha reductase inhibitor) was commenced in attempt to reduce 1,25VitD but ultimately severe hypercalcaemia (3.53mmol/L) necessitated cessation of lactation with subsequent rapid improvement of calcium levels. Initial neonatal hypercalcaemia (day 8 corrected calcium 3.08mmol/L (range 1.85-2.8)) resolved by day 14 post-partum.

Pregnancy and lactation-induced significant elevation of 1,25VitD and PTHrp postulated to be driving the PTH-independent hypercalcaemia although underlying aetiology remained under investigation; a post-partum FDG-PET was non-diagnostic. Similar biochemical and clinical pattern are well-reported in rare cases of 24-hydroxylase loss-of-function mutations. Genetic testing for CYP24A1 mutations are pending.

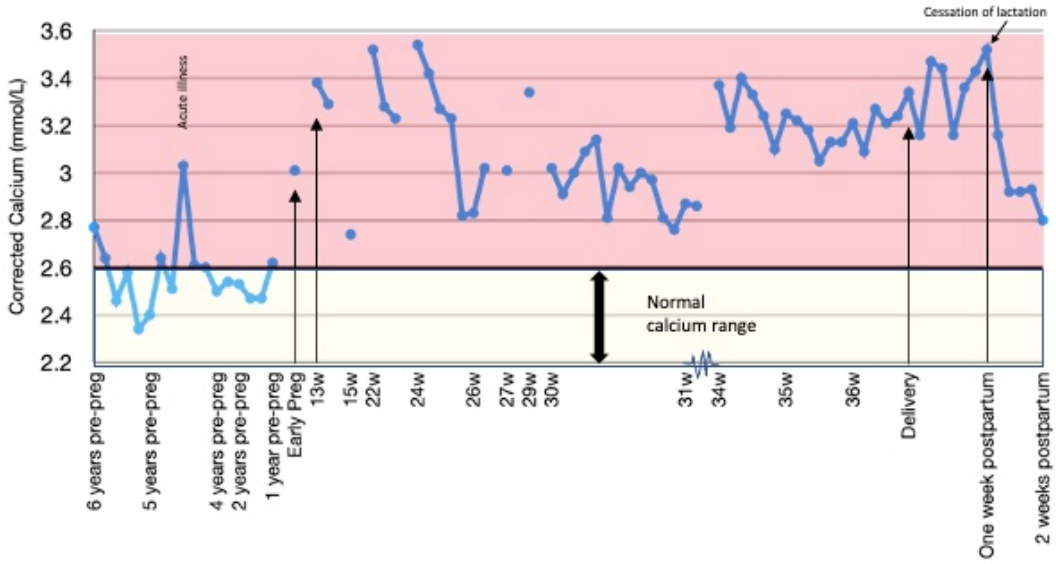
Key points:

- Differential diagnosis, investigation and management of severe PTH-independent hypercalcaemia in pregnancy and lactation
- Counselling women of child-bearing age regarding the potential course of pre-existing hypercalcaemic disorders during pregnancy
- Consideration of genetic testing for CYP24A1 mutation in long-standing undifferentiated PTH-independent hypercalcaemia with elevated 1,25VitD
- Clinical features and management of CYP24A1 mutation

FIGURE 1.

	Time period	Investigation	Results	Reference Range
1a	**2-6 years prior to conception**	Corrected calcium	2.4-2.77mmol/L (3.03 during acute illness)	(2.15-2.55)
		Phosphate	0.97-1.33	(0.75-1.10)
		UEC/LFT	Normal	
		PTH	<1 to 0.6pmol/L	(1.5-7.6)
		Urine calcium	9.9mmol/24 hours (serum calcium 2.4)	(2.5-7.5)
		Vitamin D	104-135 nmol/L	(Toxicity unlikely <150)
		1,25 Vit D	214 pmol/L	(60-200)
		PTHrp	<2pmol/L	(<2)
		Autoimmune panel	Normal	
		TSH	Normal	
		Serum EPG/IEPG	No paraprotein	
		Bone scan	Normal	
		CT chest	Unremarkable	
		Breast and pelvic USS	Nil significant	

1b **Corrected Calcium during pregnancy**



	Pregnancy	Phosphate	0.54-1.17mmol/L	(0.75-1.5)
		PTH	<0.4pmol/L	(1.6-7.2)
		PTHrp	42.27 pmol/L	(<1)
		ACE	17 U/L	(20-70)
		Vit A (second trimester)	1.7 umol/L	(1.4-4.0)

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An unusual case of syndrome of inappropriate secretion of anti-diuretic hormone (SIADH) associated with Gradenigo syndrome from acute otitis media

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Introduction:

The syndrome of inappropriate secretion of anti-diuretic hormone (SIADH) is a condition of euvolaemic hyponatraemia sometimes associated with disorders affecting the central nervous system (CNS). Gradenigo syndrome is a rare but serious complication of otitis media, defined by the triad of otitis media, severe orbito-facial pain and ipsilateral sixth cranial nerve palsy.

Case:

A 67-year-old female presented with worsening left-sided otalgia with discharge, nausea and vomiting, hearing impairment with intermittent tinnitus, and new onset diplopia. She had profuse left ear sloughy discharge, a left sixth cranial nerve palsy and CT imaging demonstrating left mastoid effusion with sclerosis at the petrous apex, which was felt to fulfil criteria for Gradenigo syndrome.

She was also severely hyponatraemic at presentation, with serum sodium 112mmol/L (135-145), serum osmolality 239mmol/kg (285-295), urine sodium 57mmol/L and urine osmolality 512mmol/kg. She was a non-smoker with a history of hypertension and dyslipidaemia. Regular medications were valsartan 80mg daily, amlodipine, atenolol and atorvastatin. She was clinically euvolaemic with normal renal function and cortisol level (470nmol/L). Incidentally, she had mild hyperthyroidism with TSH 0.2mIU/L (0.40-3.50) and free T4 26.3pmol/L (10.0-20.0), and was found to have a multinodular thyroid.

She was admitted to Intensive Care for close monitoring, intravenous broad-spectrum antibiotics and a strict 1.2 litre daily fluid restriction for management of SIADH. Valsartan was withheld. Her sodium improved gradually over 6 days and she underwent an operative left myringotomy and insertion of grommet on day 7. Her symptoms improved and she was discharged by day 13 on a prolonged course of antibiotics. Serum sodium normalised to 135mmol/L by discharge.

Conclusions:

Gradenigo syndrome is a rare entity which can cause complications that affect the CNS. Our case is the first to demonstrate acute SIADH being possibly linked with this syndrome in an adult.

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Gender affirming endocrine care at a victorian tertiary centre

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Background

Transgender and gender diverse (TGD) individuals are increasingly seeking gender-affirming care. Monash Health is one of the hospital providers for gender-affirming hormone therapy (GAHT) in Victoria. We present data from our clinic between January 2018-December 2020.

Results

173 new TGD clients were seen, mean age 27.5 years (range 17-59). The most prevalent baseline comorbidities were overweight/obesity (58% of those with recorded weight) depression/anxiety (44.5%), active or prior smoking history (33.5%), and autism spectrum disorder (8.7%).

120 (69.4%) individuals were referred for GAHT initiation. 54 (45%) commenced feminising and 44 (36.7%) commenced masculinising GAHT within six months of their initial clinic review; 22 (18.3%) did not commence GAHT, typically if individuals declined or baseline requirements (pathology, consent) were not completed.

Feminising GAHT formulations included combined oral contraceptive pill (COCP) (*n* = 23), oral oestradiol (*n* = 17), and transdermal oestradiol (*n* = 13). Androgen blockers, prescribed to 30 clients, were cyproterone acetate (CPA) (*n* = 21), or spironolactone (*n* = 9). Masculinising GAHT formulations were intramuscular (*n* = 24), transdermal (*n* = 19), and oral (*n* = 1) testosterone.

Of the 53 (30.6%) individuals referred for GAHT optimisation, 30 (56.6%) were already using feminising and 23 (43.4%) using masculinising GAHT. Feminising hormones included oral oestradiol (*n* = 17), COCP (*n* = 6), transdermal oestradiol (*n* = 3), and oestradiol implant (*n* = 1). 20 were using androgen blockers, including spironolactone (*n* = 15) and CPA (*n* = 5). Masculinising hormones included intramuscular (*n* = 20), transdermal (*n* = 2), or oral (*n* = 1) testosterone.

Discussion

Hormone therapy is an important part of gender-affirming care for many TGD individuals, and tertiary clinics provide an important service in the initiation and optimisation thereof. Further research is needed to inform evidence-based and individualised prescribing, particularly in the context of common comorbidities.

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Venous thromboembolic disease in klinefelter syndrome: a case series

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Background

Klinefelter syndrome (KS) is the most common chromosomal aneuploidy in men, occurring in 1:600 males, 80-90% of whom have a 47,XXY karyotype. Clinical features vary but include androgen deficiency and infertility. Venous thromboembolism (VTE) is also increasingly recognised, with KS patients demonstrating both a higher risk of (hazard ratio [HR] 3.95), and higher mortality from (HR 1.76) thrombotic events (1).

Context

Clinical Andrology Service, Hudson Institute of Medical Research and Monash Health, Victoria.

Cases

Ten of the 88 (11.3%) KS patients reviewed in our clinic over a 10-year period (2011-2021) have had at least one VTE event. Median age at first VTE event was 40.4 years (range 20-54); four patients had recurrent VTE. 16 VTE events were recorded during this time; the most frequent were deep vein thrombosis (*n* = 8) and pulmonary embolism (*n* = 5), with one episode each of superficial lower limb thrombus, cerebral vein thrombus, and superior mesenteric vein thrombus**.** Androgen replacement at the time of VTE was depot intramuscular (*n* = 13) or transdermal (*n* = 3). Hb prior to VTE ranged from 145-183g/L, and haematocrit ranged from 0.41-0.52**.** Additional risk factors were overweight or obesity (*n* = 5) and smoking (*n* = 3). Inherited thrombophilia work-up was performed in eight patients, one patient with superficial thrombophlebitis was not investigated, and one patient was lost to follow-up. Of those who underwent investigation, Factor V Leiden heterozygosity was identified in two cases.

Conclusion

VTE is an important co-morbidity of KS, affecting over 10% of men seen in our clinic. Further research is required regarding the nature of coagulation abnormalities that contribute to greater VTE risk in KS patients, approaches to alleviate this risk, and the role of VTE prophylaxis amongst the KS cohort.

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To block or not to block? The diagnostic and management dilemma of paraganglioma pheochromocytoma predisposition syndrome in the setting of dopamine agonist therapy

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Background

Paraganglioma pheochromocytoma predisposition syndrome is an inherited susceptibility for head and neck paragangliomas and pheochromocytomas, both of which may be malignant, in addition to renal cell cancer, thyroid cancer and gastrointestinal stromal tumour. We present the diagnostic dilemma of a *SDHB* pathogenic variant-related paraganglioma with elevated catecholamine metabolites in the setting of dopamine agonist therapy and the associated management issues of pre-operative blockade.

Case

A 79 year old lady with advanced Parkinson's disease managed with a continuous Duodopa infusion was incidentally found to have a right extra-adrenal lesion and elevated plasma 3-methoxytyramine (3-MT) 890pmol/L (RR<181) and urinary 3-MT 67.7umol/d (RR<1.3) with otherwise normal catecholamine metabolites. She underwent a laparascopic resection of the paraganglioma with a partial right adrenalectomy, yet her post-operative plasma 3-MT remained elevated at 1670 pmol/L (RR<181). She was later noted to have a new Dotatate PET avid pancreatic lesion and has since been found to have a SDHB gene causing paraganglioma pheochromocytoma predisposition syndrome.

Discussion

SDHB pathogenic variant-related paragangliomas typically secrete norepinephrine rather than epinephrine, and some can secrete dopamine. In biochemically functioning paragangliomas, the sensitivity of 24 hour urinary measurements of dopamine was 18%. This case highlights the difficulties with interpreting elevated catecholamine metabolites in a patient with phaeochromocytoma paraganglioma predisposition syndrome in the setting of continuous dopamine agonist therapy.

The US Endocrine Society recommends that all patients with a hormonally functional PPGL should undergo preoperative blockade to prevent perioperative cardiovascular complications such as an alpha-adrenergic receptor blocker. However, the current evidence suggests that adrenergic blockade is contraindicated in dopaminergic phenotype phaeochromocytoma / paragangliomas (PPGLs) due to the risk of cardiovascular collapse. This emphasises the need to revise the current perioperative management guidelines for dopaminergic phenotype PPGLs.

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Refractory Hypocalcaemia after Roux-en-Y gastric bypass surgery in a patient with pre-existing Hypoparathyroidism

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Introduction

The difficulties in managing hypoparathyroidism in a bariatric surgery patient are often under-estimated. Oral treatment with calcium carbonate and calcitriol is relatively simple but if absorption is impaired due to gastric bypass (the duodenum and jejunum are preferential sites for calcium absorption) then alternative strategies are needed.

Case:

A 37-year-old female underwent total thyroidectomy for Graves' disease in 2016 with subsequent hypothyroidism and hypoparathyroidism. After surgery she was maintained on calcitriol 0.25mcg daily and calcium carbonate 1200mg daily. In 2018 she underwent Roux-en-Y gastric bypass in the management of Stage 3 Obesity (weight 153kg, BMI 56.2kg/m2). Two years later her weight had fallen by 72kg (BMI 29.8kg/m2) and she started to develop daily symptoms of nausea and vomiting followed by lethargy and paraesthesia. She presented to our hospital with serum ionised calcium 0.93mmol/L (1.15-1.30), corrected calcium 1.97mmol/L (2.10-2.60). Treatment included oral and intravenous calcium in addition to calcitriol. On discharge ionised calcium was 1.12mmol/L. Over the following months, doses of calcitriol were slowly titrated from 0.25mcg daily to 1mcg TDS as well as calcium carbonate to 2400mg daily and cholecalciferol 5000IU per day. Despite this ionised calcium level was frequently approximately 1.00 mmol/l. Symptoms included continuing paraesthesia and demineralisation and fracture of her teeth. Normocalcaemia was only achieved after conversion of calcitriol from capsule formulation to liquid formulation at an equivalent dosage. Liquid calcitriol is difficult to obtain and expensive. Ionised calcium levels stabilised at 1.17mmol/L after 1 week of liquid calcitriol treatment.

Conclusion:

Absorption of calcium from the gut deteriorated as the patient lost more weight. This case highlights the importance of considering gastric bypass surgery carefully after parathyroidectomy especially given the difficulty in management of hypocalcaemia. Whilst escalation of doses of supplementation may be required, some patients in the literature have required reversal of their gastric bypass.

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Milk alkali syndrome in a young woman with Gastro-oesophageal reflux after Laparoscopic gastric banding

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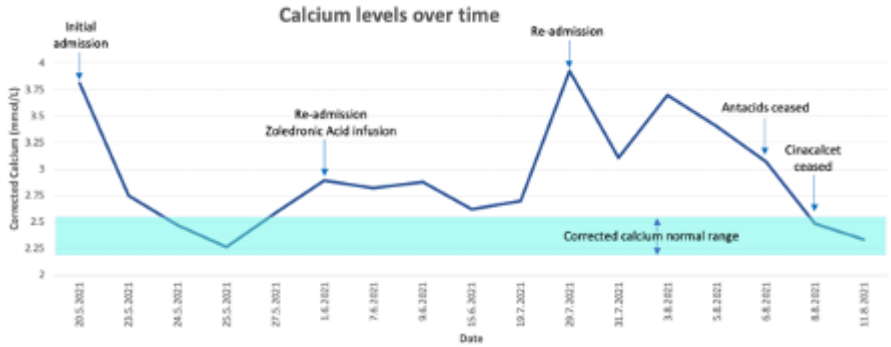
Introduction:

Milk alkali syndrome (MAS) consists of a triad of hypercalcemia, metabolic alkalosis, and acute renal failure due to ingestion of large amounts of calcium and absorbable alkali often in the form of over-the-counter antacid medications. Although MAS had become a rare presentation since the introduction of H2 antagonists and proton-pump inhibitors (PPIs) it has made a resurgence in recent years with increased use of calcium supplements for osteoporosis prevention.

Case Report:

A 35-year-old female presented to the emergency department with severe lethargy, nausea, thirst, and constipation for a few weeks. Initial bloods showed ionised calcium 1.84mmol/L [1.15-1.33], creatinine 334 µmol/L [45-85], bicarbonate 44mmol/L [20-32], parathyroid hormone (PTH) 3.5pmol/L [1.6-9.0] and vitamin D 56nmol/L [>49]. Medical history was significant for previous cholecystectomy, laparoscopic gastric banding (LAGB) and gastroesophageal reflux disease. She was taking no prescribed medications. Calcium levels normalised with intravenous fluids, but then rebounded again requiring readmission and further intravenous fluids plus 5mg zoledronic acid once eGFR >50 ml/min/1.73m^2. Cinacalcet was commenced and dose titrated to 30mg BD. Nuclear sestamibi and ultrasound did not reveal a parathyroid adenoma. Further work up including CT chest-abdomen-pelvis, ACE level and serum protein electrophoresis were unremarkable. During a third admission to hospital with recurrent hypercalcaemia, it became evident she was also taking at least 6 chewable Quick-eze® tablets (containing 750mg calcium carbonate per tablet) per day secondary to difficulty swallowing PPI tablets after her LAGB. This raised the possibility of MAS as the cause of her recurrent hypercalcaemia. On cessation of these tablets and changeover to pantoprazole for acid suppression, calcium levels normalised within 48 hours and cinacalcet was able to be ceased.



Conclusion:

Medical practitioners need to be aware of the potential adverse effects of ingesting excessive amounts of calcium carbonate and its role as a potential cause of hypercalcaemia.

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Paraneoplastic hyperthyroidism in a man with testicular cancer

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Introduction: Human Chorionic Gonadotropin (HCG) is a glycoprotein with a structure similar to TSH [1]. HCG-mediated hyperthyroidism from an HCG secreting tumour is a rare cause of hyperthyroidism [2].

Case: We report the case of a 29-year-old gentleman who presented with abdominal pain, tachycardia and transient atrial fibrillation. Abdominal CT revealed para-aortic retroperitoneal lymphadenopathy. Though no testicular mass was palpable, scrotal ultrasound revealed a testicular lesion suspected to be a primary tumour. Relevant tumour markers were elevated: HCG 682000 IU/L (<2), AFP 22 kIU/L (<11). Thyroid function tests (TFTs) were consistent with primary hyperthyroidism: TSH <0.01mU/L (0.4-4), T4 29 pmol/L (9-19), T3 7.3pmol/L (3-5.5). TSH receptor antibodies were 0.8 U/L (<1.8). The patient commenced propranolol for tachycardia and underwent right orchidectomy with histopathology confirming an 11 mm non-seminomatous germ cell tumour (pure teratoma of post-pubertal type). His tumour was exquisitely responsive to chemotherapy and 2 weeks after commencing chemotherapy, HCG decreased to 31200 U/L and TFTs normalised (TSH 0.83mU/L, T4 11pmol/L and T3 3.7pmol/L). Propranolol was ceased and the patient has had no recurrence of tachycardia or atrial fibrillation and has remained euthyroid on subsequent testing. Thionamides were not used at any stage.

Discussion

Germ cell tumours, particularly those of the non-seminoma type can produce substantial amounts of HCG [3]. In one study of disseminated non-seminomatous germ-cell tumours, hyperthyroidism was present in 3.5% of patients overall and 50% of those with HCG >50000 IU/L [2]. Hyperthyroidism will usually resolve with treatment of the tumour and reduction of HCG level. Hyperthyroidism can be the presenting feature of an HCG secreting tumour [3-6]. These tumours can be extra-gonadal, therefore absence of a testicular mass does not exclude the diagnosis [7]. HCG-mediated thyrotoxicosis should be considered in hyperthyroidism where another cause is not identified or in the presence of unusual clinical features [4].

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Hypopituitarism due to lymphocytic hypophysitis

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We report the case of a 32 year-old woman, G1P1, with lymphocytic hypophysitis and hypopituitarism diagnosed 6 months post-partum. She was admitted to the Mother and Baby Unit 6 months post-partum with anhedonia, low mood, fatigue and difficulty with attachment and bonding with her baby. During the pregnancy at 30 weeks gestation, she had been admitted with a severe headache, the cause of which was not identified at the time. At the post-partum admission, thyroid function tests done as part of a routine depression screen were suggestive of secondary hypothyroidism. Other pituitary hormones followed showing multiple deficiencies (see below). Pituitary MRI was unremarkable at this time.

	0900		0900
Cortisol	19 pmol/l (150-700)	FSH	4 U/L
TSH	2 mU/L (0.4-4)	LH	1.7 U/L
fT4	5 pmol/l (9-19	Progesterone	<1 nmol/l
fT3	4 pmol/l (3-5.5)	Oestradiol	180 pmol/l
Prolactin	13 mU/L (<340)	bHCG	<1 IU/L
GH	0.1 ug/L (<3.3)		
IGF1	64 ug/l (115-307)		

She commenced glucocorticoid and thyroid hormone replacement and started the oral contraceptive pill. At subsequent follow up, a glucagon stimulation test was arranged. Growth hormone deficiency was confirmed and she commenced daily growth hormone injections. At approximately 14 months post-partum, she decided to cease the oral contraceptive pill and fell pregnant shortly thereafter. At 11 weeks gestation in the second pregnancy, she developed gestational thyrotoxicosis when she presented with hyperemesis gravidarum. Managing the concurrent diagnoses of HCG mediated thyrotoxicosis and underlying secondary hypothyroidism proved challenging with hypothyroidism occurring with thyroxine dose reduction.

Lymphocytic hypophysitis is an inflammatory disorder of the pituitary which occurs most often in the third trimester of pregnancy or within 1 year post-partum [1]. It can present with headache, visual disturbance or symptoms of pituitary insufficiency. Though rare, it should be considered in the differential diagnosis of headaches in pregnancy and post-partum [2].

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A case of recurrent hyperparathyroidism after parathyroidectomy with autotransplantation

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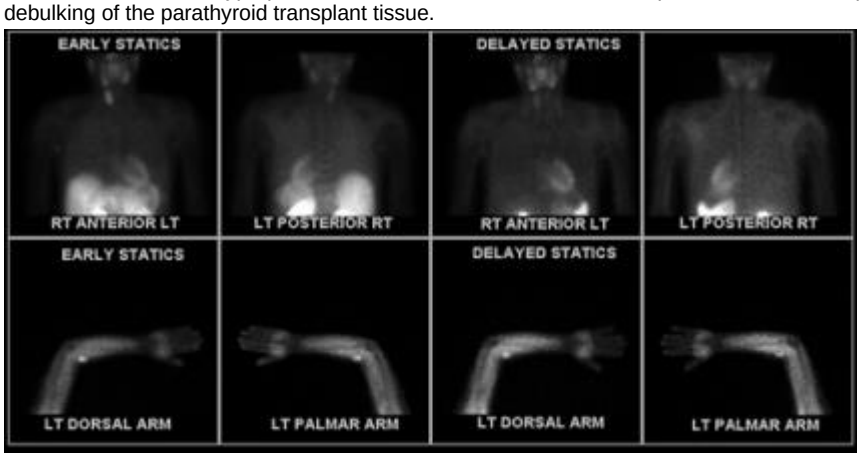
Introduction

Primary hyperparathyroidism (PHPT) is the most frequent manifestation of Multiple Endocrine Neoplasia 1 (MEN 1) developing in almost all carriers by 50 years of age. However, recurrence is reduced after near total parathyroidectomy with parathyroid auto-transplantation.

Case

A 40-year-old woman with MEN 1 underwent near total parathyroidectomy with left forearm auto-transplantation for hyperparathyroidism twenty years prior but was unfortunately lost to follow up. She was referred to the Endocrine Neoplasia Clinic for ongoing management of MEN 1 and was found to be hypercalcaemic, ionised calcium 1.41mmol/L [1.14 – 1.29], with an inappropriately normal parathyroid hormone (PTH) 3.18 pmol/L [0.32 – 8.2] with adequate vitamin D levels. She was asymptomatic. A parathyroid sestamibi scan demonstrated persistent activity in the proximal left forearm without any abnormal neck or mediastinal activity. A left/right arm study was performed to substantiate these results. Venous blood was taken from the left arm 2 minutes after tourniquet application proximal to the transplant site. Subsequently, blood was drawn from the right arm with the left arm tourniquet still in situ. The samples demonstrated a significantly elevated PTH from the left arm sample at 37 pmol/L and undetectable PTH from the right arm sample. Ultrasound of the neck and forearm demonstrated no neck abnormality but two left forearm hypoechoic masses at the transplant site; 9x2mm and 6x3mm, both with prominent vascularity. These findings were consistent with hyperplastic recurrence of PHPT in the transplanted tissue. The patient has been referred for partial debulking of the parathyroid transplant tissue.



Conclusion

We report a case of recurrent hyperparathyroidism in a parathyroid auto-transplant in a patient with MEN 1. This case highlights the importance of long-term follow-up and the approach to investigation and management when it occurs after parathyroid auto-transplantation in patients with MEN 1.

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A Hairy Case of Elevated Androgens in a Hirsute Woman

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Introduction

Differentiation of severe PCOS with elevated androgens from a virilising tumour can be difficult.

Case Presentation

A 26-year-old woman of Indian descent presented with severe hirsutism and menstrual irregularity. She had been diagnosed with polycystic ovarian syndrome (PCOS) and a left sided adrenal adenoma. She took no regular medications and no relevant family history. On examination, she had a normal BMI, no acne, temporal hair recession, acanthosis nigricans, increased muscle bulk or clitoromegaly. Ferriman-Gallwey score was 32 indicating severe hirsutism.

Initial biochemistry showed markedly elevated androgens on serial testing (see table).

Pelvic ultrasound demonstrated polycystic ovarian morphology with a left 23mm cystic lesion that was deemed “intermediate risk” (on Ovarian-Adnexal Imaging-Reporting-Data system). CT adrenals showed a left 9.3mm adrenal adenoma (29 HU) with a normal right adrenal gland. MRI adrenal findings were consistent with the CT.

24-hour urinary androgen metabolites were significantly elevated. Intravenous dexamethasone suppression test failed to suppress testosterone levels to normal range with only 25% reduction (see table). Ovarian tumour markers were within normal limits.

Biochemical Parameter	Date				
	17/4/21	9/6/21	28/6/21	26/7/21	Reference
Testosterone	4.7 nmol/L	4.3 nmol/L	4.6 nmol/L	4.8 nmol/L	<2.8
Free Androgen Index	-	28.7	24.2	-	0 – 2.9
Calculated Free Testosterone	115 pmol/L	-	-	-	1 – 34
SHBG	20	15	19	-	18 – 114
DHEAS	18.5 umol/L	-	-	-	2.7 – 9.2
DHEAS	-	-	11.4 umol/L	-	0.95 – 11.65
Androstenedione	-	-	17.6 nmol/L	-	1.5 – 10.3
17 hydroxyprogesterone	9.0 nmol/L	13.2 nmol/L	-	-	0.3 – 15.2
Cortisol	336 nmol/L	-	-	-	138 – 690
ACTH	23 ng/L	-	-	-	9 – 51
FSH	5 U/L	-	-	-	4 – 13
Oestradiol	186 pmol/L	-	-	-	88 – 607
Progesterone	<2 nmol/L	-	-	-	2 – 5
TSH	0.72 mU/L	-	-	-	0.3 – 3.5
Prolactin	311 mU/L	-	-	-	109 – 557
Normetadrenaline	220 pmol/L	-	-	-	<550
Metadrenaline	100 pmol/L	-	-	-	<447
3 methoxytyramine	<50 pmol/L	-	-	-	<181
Aldosterone	341 pmol/L	-	-	-	100 – 950
Renin	8.8 mU/L	-	-	-	3.3 – 41
Aldo/Renin ratio	39	-	-	-	<70
Sodium	140 mmol/L	-	-	-	135 – 145
Potassium	4.8 mmol/L	-	-	-	3.5 – 5.5

24 Hour Urine 9/6/21	Result	Reference
Urine Volume	1.498 L	
Excretion		
Androsterone	22.9 umol/24h	1.5 – 12.0
Etiocholanolone	17.8 umol/24h	1.5 – 12.0
5-beta 17 alpha hydroxypregnanolone	2.3 umol/24h	< 1.0
Pregnanetriol	8.6 umol/24h	0.5 – 3.5
Tetrahydro-11 deoxy cortisol	0.2 umol/24h	<0.5
Pregnanetriolone	<0.1 umol/24h	<0.5
Tetrahydro-cortisone	15.8 umol/24h	2.5 – 12.0
Tetrahydro cortisol	6.8 umol/24h	0.7 – 6.0

IV DST 28/6/21 Time	Cortisol nmol/L (138 – 690)	ACTH ng//L (9 – 52)	Testosterone nmol/L (<2.5)	DHEAS umol/L (1 – 12)	SHBG nmol/L (18 – 144)
- 60 min	469	45.4	4.78	13.4	18.6
- 5 min	422	34.9	5.24	13.2	17.3
+ 3 hr	119	6.4	3.81	10.8	19.9
+ 4 hr	79	<5	4.26	9.47	16.4
+ 5 hr	56	<5	3.28	9.99	17.9
+ 23 hr	<28	<5	4.09	6.81	22.6
+ 23.5 hr	<28	<5	3.61	6.11	19.1

The differential diagnosis in this patient is an ovarian or adrenal virilising tumour with current investigations favouring an adrenal source.

Discussion

PCOS is common and generally associated with only mild androgen excess. Both adrenal and ovarian virilising tumours are rare. Failure to suppress androgens after administration of dexamethasone is characteristic of a virilising tumour. Additionally, DHEAS

is predominantly produced by the adrenal glands and can be used as a marker of adrenal androgen secretion if the levels are elevated or do not suppress. However, differentiating between ovarian and adrenal sources can be difficult.

Conclusion

We report a case of a virilising tumour in a young woman. The abnormal imaging findings in both the left adrenal gland and left ovary have presented challenges in differentiating between these two potential sources.

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Beware asymmetric limb pain in an individual with diabetes

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The majority of individuals that present with diabetes-related foot complications have painless neuropathy, therefore pain should raise clinical suspicion for other diagnoses. Although deep-seated infections, acute Charcot arthropathy and ischaemia may present with pain, Hansen's disease can cause pain in this patient cohort, particularly given that trauma of amputation is a recognised precipitant. It is also important to differentiate the neuropathy of Hansen's disease from that of diabetes mellitus.

We report the case of a 47-year-old male of Samoan descent with Hansen's disease. This was diagnosed following increasing pain and erythematous rash post bilateral below knee amputations for necrotic feet secondary to ischaemia from severe cardiomyopathy. On day 24 post amputation, he developed a raised, erythematous rash to his right stump that was tender on palpation (*Figure 1*). Subsequently, he developed acute right fifth finger dactylitis, left eye conjunctival injection and left distal forearm swelling. The dactylitis and swelling were reported as tenosynovitis on ultrasound, and the conjunctival erythema was attributed to a dry eye.

The *Mycobacterium leprae* PCR on the skin biopsy returned positive. He was diagnosed with tuberculoid leprosy with a type 1 reaction, and commenced a 12-month course of dapsone, clofazimine and monthly rifampicin, and weaning prednisolone. The iritis, dactylitis and rash improved following treatment commencement.

Hansen's disease should be considered as a differential for diabetic neuropathy, especially when the classical distal, symmetrical pattern typically seen with diabetes is absent, or if other clinical features are present. In our multicultural society, it is important to consider Hansen's disease as timely identification and treatment prevents permanent disability. With ongoing vigilance, we may edge closer to the leprosy-free world envisioned by the WHO Global Leprosy Strategy 2016-2020. Moreover, in our patients with diabetes, we should never assume new symptoms are secondary to diabetes.

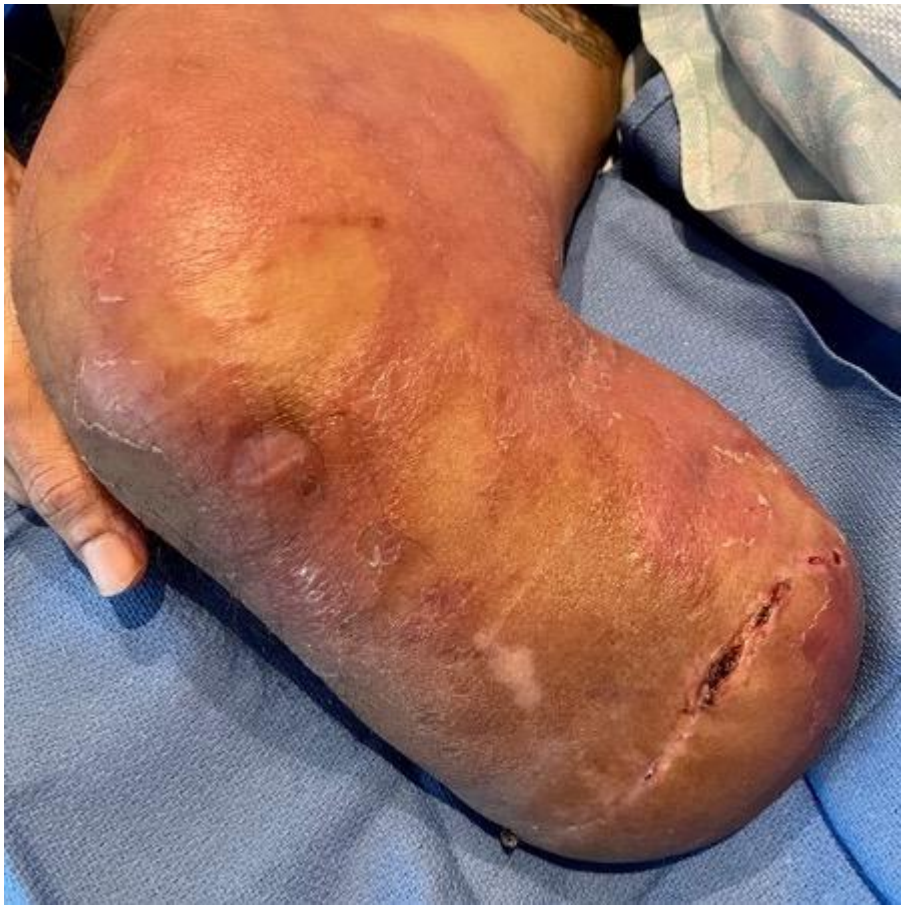

Figure 1. Rash in Hansen's disease

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What not to expect when you're expecting

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We present the case of a 29-year-old G2P1 who was reviewed at 12 weeks gestation. On examination, her BMI was 39.1 kg/m2, her blood pressure was 1280/80 mmHg and she had signs of hypercortisolism including central adiposity, dorsocervical fat pad, and acanthosis nigricans, and violaceous striae. A diagnosis of Cushing's syndrome was considered. Serial urinary free cortisol measurements were elevated at between 584-842 nmol/L (<150). Early morning cortisol was 735 nmol/L. Midnight salivary cortisol was 2.9 nmol/L (<3.0). MRI pituitary demonstrated a 3.7x4.3x3.8 mm lesion within the pituitary gland's right lobe, suggestive of a pituitary microadenoma.

Metyrapone was commenced at 18 weeks. Labour was induced at 39 weeks and complicated by post-partum haemorrhage. Metyrapone was ceased post-delivery to allow breastfeeding. Inferior petrosal sinus sampling was performed 4 months postpartum confirming elevated right side IPS to peripheral ACTH ratio. Resection of her pituitary adenoma was performed at 5 months post-partum with histopathology confirming Cushing's disease. Her postoperative course was uncomplicated other than transient diabetes insipidus. Her 48-hour post-resection and ACTH were undetectably low and she was discharged on twice-daily hydrocortisone replacement.

Discussion

Cushing's disease in pregnancy is rare due to the impairment of gonadotropin signaling in pituitary disease.[i] Changes in circulating levels of corticotrophin-releasing hormone, adrenocorticotrophic hormone, cortisol binding globulin, and CRH binding globulin make a biochemical assessment of cortisol levels in pregnancy challenging. Cushing's syndrome in pregnancy is high-risk with 60-70% of cases complicated by maternal morbidity, 50% neonatal prematurity, 25-40% neonatal mortality, and 2-4% maternal mortality. Post-pregnancy complications can include poor wound healing, osteoporosis, and heart failure. [ii] Options for treatment of Cushing's disease in pregnancy include surgical and medical therapy.

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Hypercalcaemia in granulomatous disease with response to Cinacalcet

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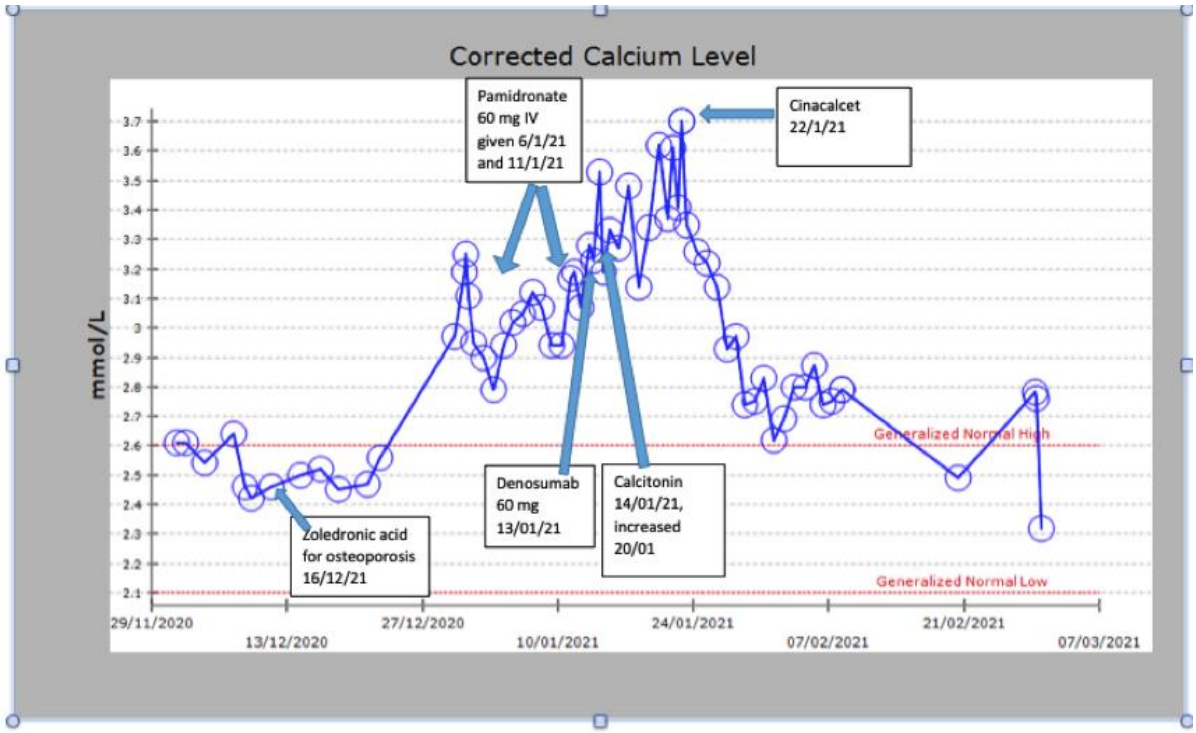
We present a case of a 71-year-old with hypercalcemia and disseminated granulomatous tuberculosis, with response to cinacalcet despite normal PTH, PTHrP, and $1,25(OH)_2$

She was diagnosed with disseminated tuberculosis disease whilst being worked up for unintentional weight loss. She was noted to be hypercalcaemic with a peak corrected calcium of 3.70 mmol/L. Myeloma screens, thyroid function, serum ACE were normal. $1,25(OH)_2D$ was 124 nmol/L (60-200) and $25(OH)_2D$ was 99 nmol/L. PTHrP was undetectable at <1.0 pmol/ (<1.05), and PTH was low at 0.6 pmol/L (1.6-6.9).

The Time course of serum calcium and management is shown in the figure. Cinacalcet 30 mg TDS was commenced with a decrease in serum corrected calcium from 3.7 mmol/L to 2.8 mmol/L within 96 hours.



Discussion

This case demonstrates an unusual picture of severe hypercalcaemia in granulomatous disease resistant to other treatment modalities but responsive to cinacalcet despite suppressed PTH and PTHrP.

Cinacalcet is a type II Calcimimetic that modulates the activity of the CaSR to increase its sensitivity to extracellular calcium and inhibit PTH release. [ii] Possible mechanisms of cinacalcet, in this case, include via PTH or PTHrP not detectable in our assay, or CaSR-mediated reduction in renal calcium absorption. .[i] [ii]

To the best of our knowledge, this is the first report of hypercalcaemia in granulomatous disease response to calcimimetics.

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Treatment resistant prolactinomas - Can we ever achieve normality?

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A 35-year-old male from home with family presented with 12 months of erectile dysfunction and morbid obesity (BMI 69). He was not taking regular medications, with no allergies. He worked as a construction worker and had a 10 pack-year smoking history. On examination, he was not Cushingoid. His blood pressure was 150/90 mmHg with no postural drop. He had bitemporal hemianopia. His testosterone level was 1.2 nmol/L with undetectable gonadotrophins and a prolactin of 73,195 mIU/L. His pituitary panel was otherwise unremarkable. MRI pituitary demonstrated an enhancing lobulated mass measuring 37x33x36 mm with right cavernous sinus and supratentorial extension, and encasement of the right internal carotid artery. He was commenced on cabergoline but his prolactin level failed to fall below 40,000 mIU/L despite up-titrating the cabergoline to 1.5 mg weekly.

Seven months later, he underwent trans-sphenoidal debulking which was limited by the fibrous nature of the tumour. Histopathology showed a pituitary adenoma staining strongly for prolactin. Surgery was complicated by central adrenal insufficiency; he was discharged on hydrocortisone replacement and cabergoline 3.5 mg weekly.

Persistent hyperprolactinaemia and field defects despite high-dose cabergoline led us to trans-cranial debulking of the remaining tumour 11 months following his diagnosis. The fibrous tumour was again difficult to resect. Surgery was complicated by right-sided cranial nerve III palsy. Histopathology again revealed an adenoma staining strongly for prolactin. His cranial nerve palsy did not improve, rendering him unemployed with significant disability. He developed hypothalamic dysfunction including uncontrollable food cravings, thermal dysregulation and lethargy, leading to a further 10 kg weight gain. Given the persistent need for high-dose cabergoline, he received six-week course of stereotactic radiotherapy, with no improvement to date in his prolactin level and visual defects. This case highlights the difficulty of managing treatment-resistant prolactinomas and their negative impact on well-being and quality of life.

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The role of SGLT2 inhibitors in achieving glycaemic control in maturity-onset diabetes of the young type 3

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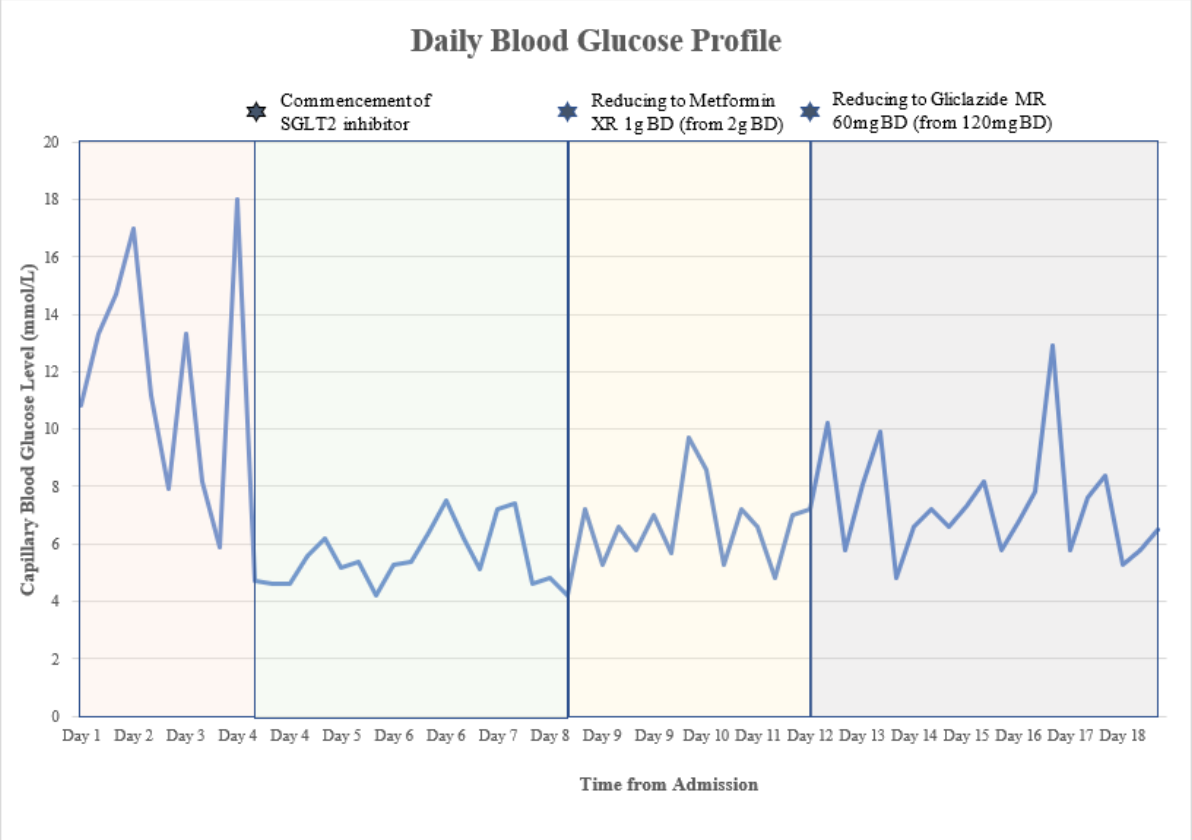
Introduction: Maturity-onset diabetes of the young type 3 (MODY3) accounts for approximately 50% of cases of MODY. First-line treatment with sulfonylureas has been well established for individuals with MODY3. In contrast, the role of sodium-glucose co-transporter 2 (SGLT2) inhibitors in the treatment of individuals with MODY3 remains unclear.

Case Presentation: A 30-year old Caucasian female was admitted with osteomyelitis and septic arthritis of the right 1st metatarsal bone and metatarsophalangeal joint in the setting of a chronic diabetic foot ulcer present for 15 months. She had a background of MODY3 treated with supramaximal gliclazide MR 120mg BD and metformin XR 2g BD At the time of her admission, C-Reactive Protein (CRP) was 27.3mg/L and an x-ray showed erosion of the 1st metatarsal head and proximal phalanx consistent with osteomyelitis and likely septic arthritis. Her HbA1C was 8.3% and she had persistent hyperglycaemia with capillary blood glucose levels (BGL) ranging from 5.9 mmol/L to 18.0 mmol/L. Empagliflozin 10mg daily was commenced in addition to her regular gliclazide MR 120mg BD and metformin XR 2g BD. Her glycaemic control immediately improved with her BGL ranging from 4.2 mmol/L to 7.5 mmol/L (Figure 1). The weighted average BGL was reduced from 8.53 mmol/L to 5.65 mmol/L after the initiation of empagliflozin, and consequently her pre-existing oral hypoglycaemic medications were reduced to the maximum recommended daily doses. Her foot ulcer improved with a reduction in pain, erythema and slough. Her CRP declined to 1.1mg/L, and she was discharged on oral Flucloxacillin for a further four weeks. Her ulcer has continued to heal, the osteomyelitis has resolved and she has now commenced mobilising with an off-loading boot.

Conclusion: This case suggests SGLT2 inhibitors may be an effective and potent treatment option in addition to sulfonylureas for individuals with MODY3.

FIGURES

Figure 1. Daily Blood Glucose Profile



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Bilateral adrenal myelolipomas: When is the right time for surgical intervention?

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Adrenal myelolipoma (AML) is a rare, non-functioning, benign tumour[1]. 70% of AMLs are small. Very few are bilateral. Myelolipomas (≥6 cm) can cause mass effects, haemorrhagic changes and resection should be considered[2].

A 56-year-old man was incidentally diagnosed with bilateral AMLs in 2017. The right-sided tumour measured 52 x 32 x 47 mm and left one was 42 x 30 x 35 mm with marked hypo-intensity -40 Hounsfield units[3]. He underwent active surveillance to avoid bilateral adrenalectomy. He was asymptomatic until the results of the most recent scan which showed a significant increase in size of right adrenal lesion. He subsequently noted bilateral flank pain over a few weeks. On examination, he was normotensive and clinical features did not suggest Cushing's syndrome. The adrenal lesions were not palpable.

Plasma metanephrines, renin to aldosterone ratio and DHEAS were normal, suggesting non-secreting tumours. There is a theory that elevated ACTH may drive the growth[4], but ACTH was not raised. Addison's disease and congenital adrenal hyperplasia (CAH)[5] were excluded. 1 mg dexamethasone suppression test excluded Cushing's syndrome. Repeat CT in 2018 revealed an increase in size of right lesion with a stable left one. In 2021, the right-sided lesion further grew bigger 73 x 65 x 82mm, despite stable left mass (Figure 1).

There are no specific guidelines for management of AML and management is individualised. Given significant growth rate (maximum diameter up to 8.2 cm), it was decided at the endocrine radiology meeting that, it would be reasonable to remove right adrenal lesion with ongoing monitoring of left side radiologically. Risk of left adrenal haemorrhage is low. Currently, he is awaiting surgical review.

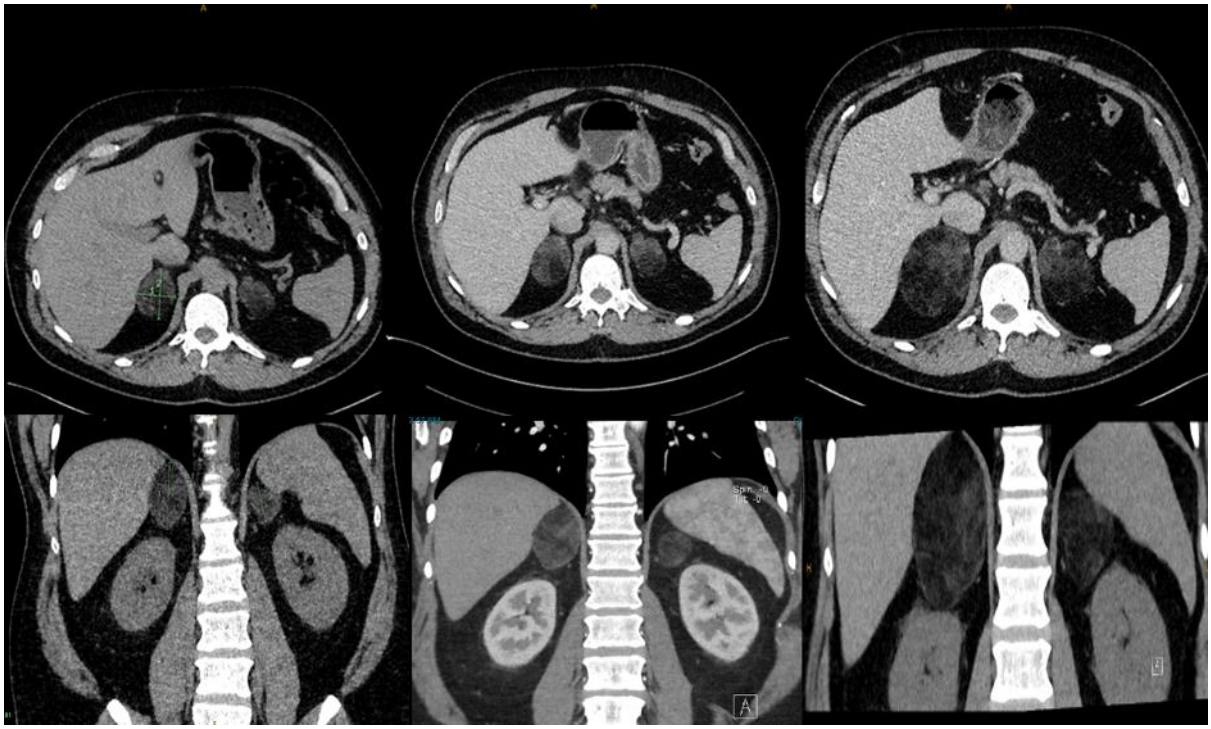

Figure 1: CT (Right to left) right adrenals: 52 x 32 x 47 mm (2017), 54 x 31 x 59 mm (2018), 73 x 65 x 82mm (2021).

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A clinical case of Graves' oscillations

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Stimulation of the thyroid epithelial cells by thyroid hormone receptor antibodies (TRAb) in Graves' disease, usually leads to hyperthyroidism[1]. TRAb is crucial not only in the diagnosis but also for the prognosis and management[2]. TRAb can be functionally characterised by bioassays. We present a case of "antibody switch" resulting in challenging management.

A 60-year-old woman presented with thyrotoxicosis in January 2018. She had a diffuse goitre without eye signs or brisk reflexes. Her thyroid function tests were consistent with thyrotoxicosis. Both elevated TRAb and diffuse uptake on the technetium scan support Graves' disease. Thyroid ultrasound showed mildly enlarged thyroid gland with increased vascularity without nodules. Carbimazole 10 mg tds was commenced, causing biochemically hypothyroid, 6 weeks later. She stabilized on carbimazole 15 mg/day. In December 2018, she had mild orbitopathy despite normal TRAb with euthyroidism. Later, she was well on carbimazole 5 mg/day.

In October 2020, she developed significant fatigue. Surprisingly, TRAb was highly positive but she was hypothyroid clinically and biochemically. Carbimazole was stopped. Thyroxine 100 mcg per day was started. Because of discordant results, heterophile Ab and dilutional studies were excluded. TRAb positivity was again confirmed by thyroid-stimulating immunoglobulin (TSI). All results are in table 1.

2 months later, she became hyperthyroid and thyroxine was reduced to 300 mcg/week with good response. TRAb and TSI remained elevated. Functional characterization of TRAb was requested using Thyretain assay. Blocking antibodies was positive (71.8%), triggering "Ab switch"[3].

Presence of predominantly blocking antibodies resulted in hyperthyroidism switching to hypothyroidism and is potential for further oscillations. Block and replace regimen can be considered but this requires close monitoring[4]. Definitive therapies are preferred for stability of thyroid function. Radioactive iodine is not ideal given Graves' orbitopathy. Our patient had total thyroidectomy without complications and is currently euthyroid on thyroxine.

Year	Free T4 9.0 – 19.0 pmol/L	Free T3 2.6 – 6.0 pmol/L	TSH 0.4 – 4.0 mIU/L	TRAb <2.1 IU/L	TSI (active Graves' >0.55 U/L)	Treatment
Jan 2018	29.6	14.5	<0.008	8.7		Carbimazole 10 mg tds
Feb 2018	2.5	2.7	17.3	27.9		Carbimazole 5mg bd
May 2018	11.4	6	0.09	20.4		Carbimazole 15 mg/day
Dec 2018	9.1	4.6	0.75	<0.8		Carbimazole 10 mg/day
April 2019	12.1	4.8	0.31	<0.8		Carbimazole 15 mg/day
Aug 2019	11.2	4.4	7.3			Carbimazole 10 mg/day
March 2020	11.4	4.2	1.6	<0.8		Carbimazole 5 mg/day
Oct 2020	<5.4	<2.3	70.7	88.5		Carbimazole ceased/Thyroxine 100mcg/day
Dec 2020	17.7	5	0.08	74.9		Thyroxine reduced to 300mcg/week
Feb 2021	12.1	4	2.07	207	>40	Thyroxine 300mcg/week

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A Hairy Affair

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Background

Adrenocortical carcinoma (ACC) is a rare, aggressive malignancy with reported incidence of 1-2 cases per 1 million population[2]. Those who present with hormonal excess have concurrent hypercortisolism and hyperandrogenism. Adrenal tumours that solely secrete androgens are extremely rare (less than 10% of secretory ACCs) and produce hirsutism, virilisation and amenorrhoea in the majority of patients[1].

Case Presentation

A 34 year old previously well woman presents with rapidly progressive hirsutism, acne and secondary amenorrhoea. Initial investigations showed elevated testosterone levels (total testosterone 3.9nmol/L [RR: 0.5-2.0nmol/L]) free androgen index (FAI) 17% (RR: 0.4-6.0%) and she was commenced on the oral contraceptive pill. Two months later in the setting of dyspnoea and chest tightness, a V/Q scan revealed unprovoked bilateral pulmonary emboli. A subsequent CT chest and abdomen revealed bilateral occlusive segmental pulmonary emboli with pulmonary infarcts as well as a left adrenal lesion measuring 125mm x110mm x140mm with invasion of the left renal vein and inferior vena cava. There was also paraaortic adenopathy and hepatic metastases.

Further biochemical investigations revealed normal plasma and urinary metanephrines, aldosterone renin ratio, 24 hour urinary free cortisol and chromogranin A levels. Repeat androgen studies showed a total testosterone of 5.5 nmol/L and FAI 14.9%. DHEAS was >27umol/L (RR 1.8-9.2umol/L). These results were suggestive of a pure androgen secreting adrenal tumour. A liver biopsy showed histopathology results consistent with metastatic adrenocortical carcinoma.

Given stage 4 disease with invasion of the left renal vein and inferior vena cava, surgical management was not offered. She was started on chemotherapy and mitotane in addition to hydrocortisone. The development of a pruritic morbilliform rash two weeks after starting mitotane has limited its escalation in dose.

Conclusion

Pure androgen secreting ACCs are very rare. Those who present with rapid onset hirsutism should be screened for an underlying androgen secreting tumour.

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Vaccine induced autoimmune thyroiditis resulting in Hoffmann Syndrome and pseudorhabdomyolysis

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Hoffmann syndrome is a rare form of hypothyroid myopathy characterised by muscle weakness, stiffness and pseudohypertrophy. Here we report an otherwise healthy 31 year old male (BMI 24) who presented with progressive headaches, myalgias, muscle fatigue, dry skin, mental lethargy, weight gain of 5 kg within 3 months following administration of the influenza vaccine. Examination revealed temporal muscle hypertrophy, reduced relaxation of deep tendon reflexes without neck swelling or masses. Further investigations revealed markedly elevated TSH (220), Creatinine (156), Creatine kinase (2537), TSH-receptor antibodies and thyroid peroxidase antibodies with reduced free thyroxine levels (3) and eGFR (50). Following introduction of supplemental thyroxine therapy (100mcg OD) the patient's symptoms improved and serum levels of Creatine and creatinine normalised. Thyroid function rebounded to transient hyperthyroidism, resulting in a short course of Carbimazole (10mg OD) which was progressively weaned off until the patient's antibody levels and thyroid function normalised. This is the first case of vaccine induced Hoffmann syndrome reported in the literature.

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Gestational thyroid storm with pulmonary hypertension in setting of molar pregnancy

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Case study:

Mrs AT is a 49-year-old Sudanese G8P7 lady who was diagnosed with thyroid storm after presenting with symptomatic heart failure, severe hypertension, tachycardia, nausea and vomiting. Her medical history was significant for obesity and non-pharmacologically managed hypertension. Investigations revealed highly elevated βhCG levels >239,000IU/L, prompting a pelvic ultrasound that confirmed presence of hydatidiform molar pregnancy. She was found to be newly thyrotoxic with thyroid stimulating hormone (TSH) <0.03mIU/L (0.38–5.1mIU/L), free T4 of 60.4pmol/L (8.0– 16.5pmol/L) and T3 of 20.2pmol/L (3.3–6.8pmol/L). TSH receptor antibodies were negative for Graves' disease. A transthoracic echocardiogram showed severe pulmonary hypertension with estimated pulmonary artery systolic pressure >60mmHg and moderate-severe tricuspid regurgitation. She was managed for thyroid storm with propylthiouracil (PTU), intravenous hydrocortisone, Lugol's iodine solution and high-dose beta blockers, with concerns surrounding high peri-operative risk. Evacuation of the molar pregnancy was performed via dilation and curettage. Pathology confirmed complete hydatidiform mole. Post-operatively, βhCG levels declined rapidly with associated improvement in thyroid function. However, Mrs AT remained thyrotoxic requiring ongoing management with PTU post-operatively.

Discussion:

Molar pregnancy typically presents with vaginal bleeding, excessive uterine enlargement and hyperemesis gravidarum. Thyroid storm is a rare presentation for molar pregnancy, but early identification and treatment can significantly improve morbidity and mortality.

Hyperthyroidism in molar pregnancy is attributed to highly elevated βhCG levels, which increase free T3 and T4 through stimulation of the TSH receptor due to structural similarity between βhCG and TSH.

Following molar pregnancy evacuation, it can take months for βhCG levels to become undetectable and thyroid function to normalise. Pulmonary hypertension can be reversible with treatment of the underlying thyroid disease. Therefore, we anticipate resolution of pulmonary hypertension and heart failure in Mrs AT following normalisation of her thyroid function.

Conclusion:

Molar pregnancy should be considered in women of reproductive age who present with thyrotoxicosis.

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Case report: erythropoietin for successful management of postural hypotension secondary to diabetic autonomic neuropathy

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INTRODUCTION

Postural hypotension is a debilitating manifestation of diabetic autonomic neuropathy that is difficult to treat. It’s thought to be secondary to damaged sympathetic fibres and poor baroreceptor response[1]. We report a case of postural hypotension successfully managed with erythropoietin in a young woman with type 1 diabetes mellitus.

CASE

A 24-year-old woman with symptomatic postural hypotension on a background of type 1 diabetes for 17 years presents to Endocrinology clinic for evaluation. Whilst past glucose control had not always been ideal (HbA1c up to 12%), more recent reports were commonly <7.0%. Diabetes complications included retinopathy, painful peripheral neuropathy, and gastroparesis.

Onset of postural hypotension was relatively abrupt and prompted hospitalisation in May 2019. Supine BP 121/84 dropped to 80/64 upon standing, improving to 98/73mmHg after 5mins. Complete blood count, serum electrolytes, renal and liver function tests, inflammatory markers, cortisol, and ACTH were unremarkable.

She trialled multiple different agents including: fluodrocortisone, dothiepin, bethahistine, caffeine, midodrine, and octreotide. Compression stockings as recommended by vascular occupational therapist were inappropriate due to exacerbation of painful peripheral neuropathy.

Nerve conduction studies in July 2019 and May 2020 confirmed a length-dependent axonal sensorimotor large fibre peripheral neuropathy without demyelination.

In February 2021, she commenced weekly erythropoietin 6000U. She did not have anaemia (Hb 126 [ref: 115-160]) or renal impairment (creatinine 62). Her symptoms had significantly improved on subsequent review, with resolution of postural drop (sitting BP 129/89; standing BP 125/87; 3min standing BP 124/89). Follow up Hb was 156.

CONCLUSION

This case report is an example of erythropoietin successfully managing postural hypotension secondary to diabetic autonomic neuropathy. There is sparse literature on this topic, with postulation that erythropoietin’s pressor effect is from increased blood viscosity with increased haemoglobin production[2], or increased nitric oxide binding reducing vasodilatation of vasculature[3]. Further research is required into this phenomenon.

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Hypoglycaemia due to metastatic insulinoma in an insulin-dependent type 2 diabetic patient successfully treated with Lutate

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Background:

Insulinoma is exceptionally rare in a patient with pre-existing diabetes but important not to miss as a cause of debilitating hypoglycaemia. Managing recurrent hypoglycaemia in metastatic unresectable insulinoma is extremely challenging given lack of definitive surgical cure, poor prognosis and limitations of available options e.g. paucity of data, modest efficacy and patient intolerance.

Case Presentation:

We describe a 90-year-old man with insulin-dependent type 2 diabetes mellitus (T2DM) who, despite insulin cessation, presented with recurrent hypoglycaemia (2.5 mmol/L) associated with confirmed inappropriate endogenous hyperinsulinaemia (24 µIU/mL). Sulfonylurea use and insulin antibodies were excluded. CT abdomen detected three large arterially-enhancing liver lesions. DOTATATE PET/CT scans showed increased activity in the pancreatic tail and extensive liver metastases. Liver biopsy confirmed well-differentiated metastatic neuroendocrine tumour. He was unsuitable for surgical resection and failed Octreotide therapy. Four cycles of Lutate resulted in hypoglycaemia resolution and he is currently well 5-years after Lutate with sustained clinical, biochemical and radiological response.

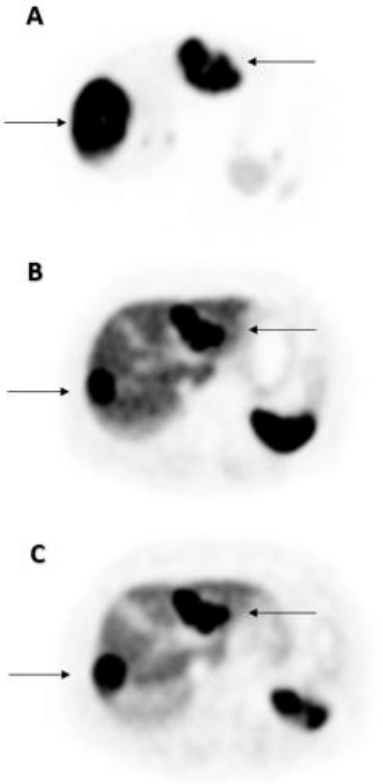
Discussion:

Literature review yielded 13 cases of metastatic insulinoma in pre-existing diabetes. Majority had T2DM (mean age 59 years at diagnosis). The most common primary site was pancreatic tail with liver being the most common site of metastases. Lutate has not otherwise been reported in a diabetic patient with metastatic insulinoma. Treatment outcomes were mixed. Literature review revealed 33 cases of Lutate in metastatic insulinoma. Vast majority had positive outcome with interpretation limited by publication bias and short follow-up.

Conclusions:

This case is unique due to the paradoxical entity of insulinoma in insulin-dependent diabetes and positive sustained outcome after Lutate despite poor expected prognosis. Lutate is a potential effective and well-tolerated treatment option in unresectable metastatic insulinoma with benefits including hypoglycaemia resolution and reduction in metastatic burden. However given scarce data, further controlled studies exploring Lutate efficacy in these patients is warranted.

FIGURE 2 – 68Ga-DOTATATE MIP images pre- and post-Lutate therapy



Axial-view maximum intensity projection (MIP) images showed interval reduction in size of both dominant hepatic metastases pre-Lutate (A) to 1.5 years post-Lutate (B), which remained stable at 3 years post-Lutate (C). Physiological uptake in the spleen and the remainder of the liver is also visualized.

A pharmacological Conn artist: The hazards of licorice in complementary medicine

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Background

Glycyrrhizin glabra, licorice root, is an ingredient in traditional herbal remedies and confectionery products. Its main active ingredient, glycyrrhizin, has anti-inflammatory and antioxidant effects,(1) but also has a potentially hazardous association with pseudohyperaldosteronism.(1)

Case

A 17-year-old girl presented to the emergency department with chest pain, nausea and tremors. She had been suffering from intermittent nausea and abdominal pain for two years but had no other medical conditions, no regular prescription medication, and denied any substance abuse. Physical examination was unremarkable except for a tachycardia of 110 beats per minute with a blood pressure of 102/62mmHg. Blood tests revealed hypokalaemia 2.8mmol/L (3.6-5.2mmol/L), elevated lactate 8.7mmol/L (0.5-2.2mmol/L), and leukocytosis 17.9 x 10^9/L (4.0-12.0 x 10^9/L). Serum aldosterone and renin were 151pmol/L and 28pmol/L respectively, with an aldosterone-renin ratio of 5.4.

Further history revealed she had taken "*GIT Regenex*", a herbal remedy containing *Glycyrrhizin glabra*, for ten days prior. Dosing instructions for this remedy recommended a daily glycyrrhizin intake of 157.34mg, exceeding the daily maximum of 100mg recommended by the World Health Organisation. She responded well to oral potassium and intravenous magnesium replacement overnight and was discharged home. A safety report was sent to the Therapeutic Goods Administration for GIT Regenex.

Discussion

Intestinal bacteria convert glycyrrhizin to 3-monoglucoronyl-18-glycyrrhetinic acid and glycyrrhetinic acid (GA).(2) GA inhibits the 11β – hydroxysteroid dehydrogenase 2 enzyme in the distal nephron, decreasing conversion of cortisol to cortisone, and increasing mineralocorticoid receptor activation through cortisol binding.(2) This causes kaliuresis and fluid retention similar to primary aldosteronism, but with normal or low serum aldosterone and renin.(3) At least 16 individual cases and 18 clinical trials have described weakness, hypertension, and even ventricular arrhythmias associated with licorice since 1970.(3-18)

Conclusion

Our case highlights the need to consider licorice-induced pseudohyperaldosteronism caused by complementary therapies in the differential diagnosis of hypokalaemia.

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A TRAb for young players thyroid autoantibodies in chronic lymphocytic leukaemia

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We present the case of an 80-year old female with weight loss secondary to Graves' thyrotoxicosis. This patient had chronic anxiety, increasing myopathic symptoms which impacted on her Irish dancing and mild bilateral orbitopathy. On further history she has a 13 year history of chronic lymphocytic leukaemia (CLL) not requiring treatment to date. This is on a background of post-menopausal osteoporosis without fractures. Progressive bone loss was seen despite treatment with zoledronic acid and calcium/vitamin D supplementation, likely contributed to by a degree of hyperthyroidism. Chronic lymphocytic leukaemia (CLL) is well known to be associated with autoimmune phenomena. These are most frequently related to the haematopoietic system but other autoimmune disorders, including rheumatoid arthritis, pernicious anaemia, myasthenia gravis and Graves' disease have also been reported in patients with CLL [1]. In this case discordance is seen between the high TRAb levels and the near normal thyroid function tests suggesting the presence of TSH receptor blockade (Table 1.). This case highlights the need to be mindful of antibodies beyond the TRAb when considering autoimmune thyroid dysfunction, in this case, possibly associated with an underlying B-cell leukaemia.

Date	February 2020	June 2020	September 2020	March 2021	August 2021
TSH mIU/L (0.4-5.0)	5.94	2.07	0.19	3.64	0.39
fT4 pmol/L (10-20)	11.0	12.8	18.4	14.3	15.9
fT3 pmol/L (2.3-5.7)	4.8	3.5	4.3	4.4	5.1
TRAb IU/L (<2.1)	59.3	77.2	43.2	49.1	48.8

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Interference in oestradiol immunoassay results a role for liquid chromatography-mass spectrometry

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We present a case of a 60 year-old female treated for metastatic, oestrogen receptor positive breast cancer. She was referred to endocrinology for persistently elevated oestradiol levels raising her oncology team's concern of disease progression. Medications included ribociclib (CDK 4 and 6 inhibitor), goserelin (GNRH agonist) and fulvestrant (oestrogen receptor down regulator). Hormone profile showed oestradiol 530 pmol/L, LH 0.1 IU/L, FSH 6.5 IU/L. Given the discordant picture between sex hormone axis blockade and high levels of oestrogen, repeat testing with liquid chromatography-mass spectrometry (LC-MS) was performed. This showed an expected low oestrogen level at 11 pmol/L.

Oestradiol is synthesised by aromatisation of androgens, catalysed by the enzyme aromatase. In healthy women of reproductive age this primarily occurs in the ovaries. Additionally, oestrogens can be produced peripherally by aromatisation of androgens, adipose tissue being a major site. Oestradiol has conventionally been measured by immunoassays which have only modest sensitivity (lower limits of quantification 100 – 350 pmol/L)[1]. In oestrogen receptor positive breast cancer a number of drug classes are used to reduce hormone exposure to malignant cells. Fulvestrant competitively binds to oestrogen receptors with consequent downregulation. Fulvestrant is known to interfere with immunoassays giving falsely elevated levels[2]. This case highlights the need to be mindful of interference when measuring oestradiol and send for LC-MS if clinically warranted.

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Craniopharyngioma with early recurrence insuring remission

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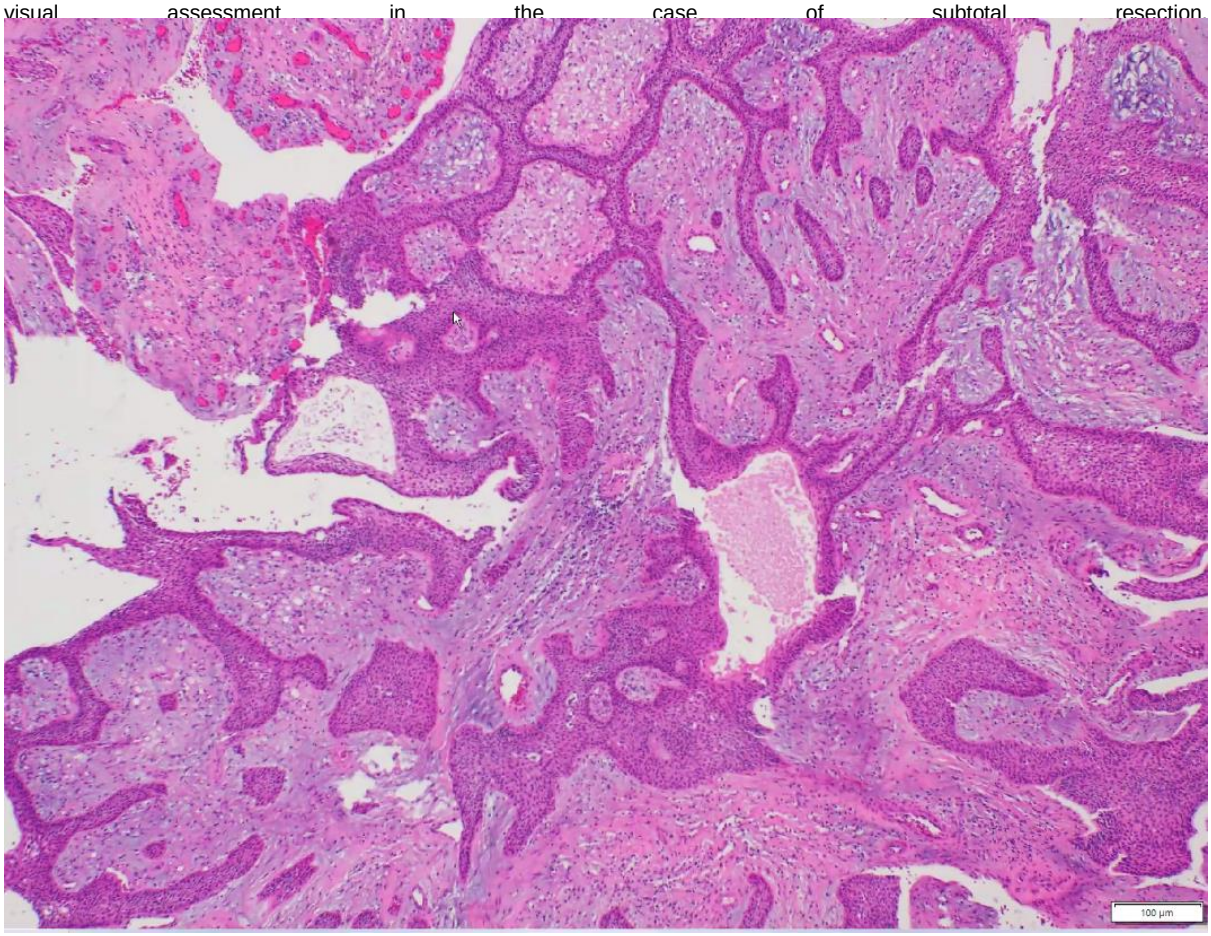
A 27 year-old male overseas student presented to his GP with new headaches and blurred vision, developing over a two month period. Bilateral inferior quadrantanopia was identified and a 13.8 x 12.3 x 12.6mm solid/cystic suprasellar mass was seen on MRI inseparable from the optic chiasm. No endocrine dysfunction was present. Transcranial pituitary surgery successfully debulked the optic apparatus in a subtotal resection. Replacement thyroxine, hydrocortisone and desmopressin were required postoperatively and vision improved. Histopathology showed papillary craniopharyngioma positive for BRAF V600E with Ki-67 <2%. Attempts were made to secure a BRAF/MEK inhibitor to delay repeat intervention however the patient's insurer was not forthcoming. An MRI at three months showed rapid tumour re-growth at 26.0 x 29.0 x 22.6mm with new cystic components compressing the optic apparatus and new extension into the third ventricle. The patient was reluctant to proceed to further surgery however this was initiated after an acute deterioration in vision. Gross total resection was achieved without complication via trans-sphenoidal approach and vision again stabilised.

This case exhibits an aggressive phenotype of craniopharyngioma with significant threat to vision. Given the patient's young age attempts were made to treat with targeted BRAF/MEK which have shown promise in the treatment of BRAF V600E positive craniopharyngiomas[1] though this was not possible. This case highlights the need for close follow up with interval imaging and visual assessment in the case of subtotal resection.



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Euglycaemic ketoacidosis post COVID vaccination in patient with diabetes mellitus on SGLT2 inhibitor

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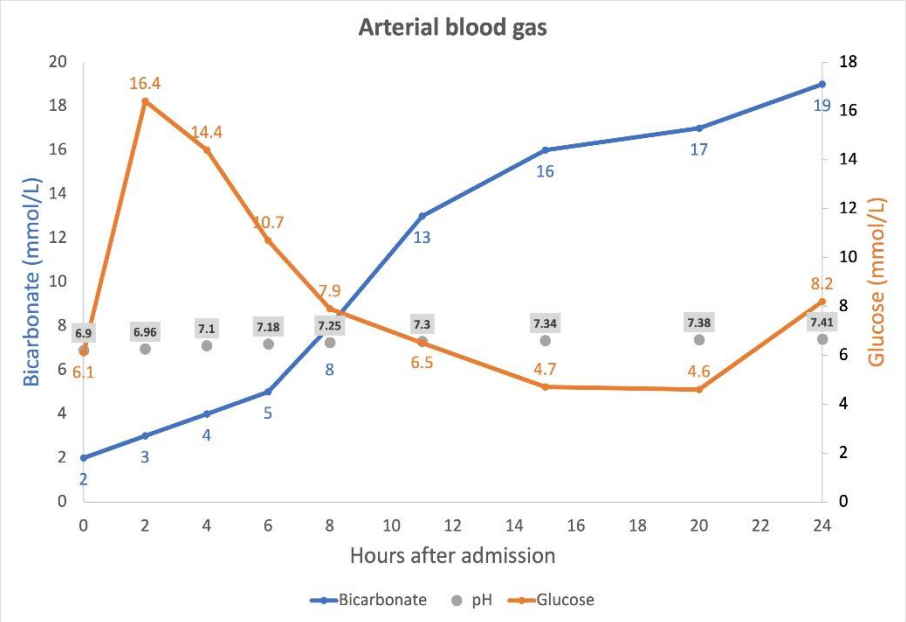
Case presentation

58-year-old male with type 2 diabetes mellitus on empagliflozin, gliclazide and linagliptin presents to emergency department with 1 day of nausea, vomiting and upper abdominal pain. He was started on empagliflozin 4 days prior and received first dose of Oxford-AstraZeneca vaccine 2 days prior. Bloods showed severe metabolic acidosis, BGL 6.1mmol/L, ketones 3.8mmol/L, lactate 2.2mmol/L and Hba1c 11.2%. Inflammatory markers were normal. Transferred to ICU for insulin infusion and intravenous fluids. After resolution of euglycaemic DKA patient was discharged home on ryzodeg insulin, linagliptin and gliclazide. Islet cell antibodies were negative making diagnosis of LADA or type 1 diabetes unlikely. On follow up one month later patient remained insulin dependent.



Discussion

Euglycaemic DKA (euDKA) is rare but serious complication associated with SGLT2 inhibitors use in patients with diabetes mellitus. Common triggers include prolonged fasting, low caloric intake, alcohol use and intercurrent illness. COVID-19 infection causes euDKA in patients on SGLT2 inhibitors[1]. SARS-CoV-2 utilises the ACE-2 receptor to enter islet cells leading to direct cellular destruction. The decrease in insulin production predisposes to euDKA while acidosis also favours entry and replication of the virus[2].

We report the first case of euDKA shortly after administration of a COVID-19 vaccine. The Oxford-AstraZeneca vaccine uses a viral vector to deliver antigen coded genetic material and does not contain any live virus. MEDLINE search reveals one case report of HHS and newly diagnosed diabetes post Pfizer vaccine[3]. COVID vaccines have also been shown to induce transient hyperglycaemia[4]. SGLT2 inhibitors does not confer significant protection during COVID-19 syndrome[5]. Reactions to COVID vaccines are common and include gastrointestinal symptoms such as nausea and reduced intake[6]. Clinicians need to be vigilant for development of euDKA and it may be necessary to advise withholding SGLT2 inhibitors two days prior to receiving COVID vaccine and to self-monitor BGLs.

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Rapid development of Cushing's syndrome following peptide receptor radionuclide therapy with 177Lu-DOTATATE in a case of metastatic pancreatic neuroendocrine tumour

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Background: Lutetium 177 (^{177}Lu) - DOTATATE is a form of peptide receptor radionuclide therapy (PRRT) utilized in the treatment of neuroendocrine tumours. Carcinoid and catecholaminergic crises have been previously described following PRRT. There have been no previous reports on ^{177}Lu-DOTATATE-induced Cushing's syndrome.

Case Description: A 55-year-old female with asymptomatic, well-differentiated, Grade 3 metastatic pancreatic neuroendocrine tumour previously treated with lanreotide and carboplatin/etoposide underwent first cycle PRRT with ^{177}Lu-DOTATATE. Five days thereafter, she presented to hospital with rapid Early morning cortisol was elevated 1042nmol/L (185 – 624nmol/L), as was ACTH 411ng/L (7.2 – 63ng/L) and 24hour urinary free cortisol 9834nmol/day (60-305nmol/day). Cortisol did not suppress on 1mg dexamethasone suppression test (DST) at 1209nmol/L or on 8mg DST (cortisol 1444nmol/L; ACTH 462ng/L). Subsequent repeat 24hour urinary free cortisol five days following the initial revealed further elevation at 16,237nmol/day (60-

305nmol/day). She was also hypokalaemic 2.9mmol/L (3.5-5.2mmol/L). Pre-PRRT serum potassium was repeatedly normal but cortisol and or ACTH were not measured. The patient was diagnosed with ectopic ACTH-dependent Cushing's syndrome and was commenced on metyrapone and octreotide therapy leading to rapid reduction in plasma cortisol and ACTH levels. Retrospective immunohistochemistry of a liver metastasis revealed ACTH staining.

Summary: We report, for the first time, a patient with asymptomatic apparently non-functioning pancreatic neuroendocrine tumour, who rapidly developed symptomatic ectopic ACTH-dependent Cushing's syndrome after ^{177}Lu-DOTATATE therapy.

Conclusions: This case raises the possibility that akin to other hormonal crises, PRRT can trigger marked ACTH release from neuroendocrine tumour tissue, rapidly leading to clinical manifestations of Cushing's syndrome. Whether the rapid improvement in ACTH and cortisol post PRRT was due to medical therapy, and/or due to offset of PRRT-associated 'tumour lysis' remains uncertain.

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5-alpha-reductase inhibitor therapy in a case of Kennedy's disease

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Background: Kennedy's disease or spinal and bulbar muscular atrophy is an X-linked condition caused by a trinucleotide repeat expansion of a CAG repeat in the first exon of the androgen receptor gene. This mutation leads to an increased number of glutamine residues in the amino-terminal domain of the receptor. Androgen receptors are expressed widely in the brain and anterior horn cells of the spinal cord. It is hypothesized that the presence of these abnormal receptors is toxic to motor neurons which results in progressive bulbar and extremity muscle weakness. The androgen receptor mutation also results in partial androgen insensitivity. There are no specific therapies for Kennedy's disease, but 5-alpha-reductase inhibitors which block the conversion of testosterone to the more potent dihydrotestosterone (DHT) have been reported to possibly lead to functional improvement.

Case Description: A 69-year-old man with a fifteen-year history of Kennedy's disease functionally affected by swallowing difficulties, speech impairment and weakness of proximal arms and legs began treatment with 5-alpha-reductase inhibitor Dutasteride. He also had manifestations of androgen insensitivity with gynaecomastia and decreased sexual function. Prior to commencement of Dutasteride, his total testosterone level was 23.9nmol/L (6.0 - 28.0nmol/L), SHBG 75nmol/L (15-50nmol/L) and DHT 0.1nmol/L (0.4 – 2.5nmol/L). His creatine kinase was 256U/L (<201U/L). Following two years of treatment with Dutasteride, total testosterone was 22.6nmol/L (6.0 - 28.0mmol/L, DHT <0.1nmol/L (0.4 - 2.5nmol/L) and CK 380U/L (<201U/L). Subjectively, the patient reported improvement in his proximal upper and lower limb muscle strength but no change to his hypoandrogenism symptoms.

Summary: We report the case of a patient with Kennedy's disease treated with 5-alpha-reductase inhibitor therapy. Total testosterone levels remained in the normal male reference range but with minor reduction of DHT levels. There was some subjective improvement in motor neuron symptoms but no change to manifestations of androgen insensitivity.

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Adrenal ganglioneuroma – a case of a rare benign entity with atypical imaging features

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Adrenal incidentalomas are commonly detected and their assessment is a clinical scenario frequently encountered by endocrinologists. Investigation to exclude autonomous hormonal secretory function and malignant potential is essential, and often guides management. Characterising adrenal masses using contrast-enhanced CT utilises understanding of typical perfusion pattern of benign adenomas. Features indicating malignant risk include large size, calcifications, irregular margins and necrosis.[1]

The case presented is a 43-year-old female with an incidentally detected right adrenal mass. Background includes obesity (BMI 39kg/m^2), bicuspid aortic valve and recently diagnosed hypertension, controlled on amlodipine and perindopril. Family history includes maternal Ehlers-Danlos syndrome and is negative for malignancy. Investigations revealed no excess secretion of cortisol, aldosterone, androgens or catecholamines, normal renal function and normokalaemia. Imaging demonstrated a 50mm heterogenous mass, with punctate focus of calcification, and washout characteristics not typical of a lipid-rich adenoma. There was no evidence of metastatic disease on 18F-FDG PET, but the lesion was FDG-avid. Imaging features raised concern for adrenocortical carcinoma. Adrenalectomy was performed laparoscopically, with successful en-bloc resection and no evidence of invasion into surrounding structures. Recovery was uncomplicated. Histopathology revealed a benign adrenal ganglioneuroma.

Adrenal ganglioneuromas are benign differentiated neoplasms which arise from neural crest cells and mostly contain Schwann cells, ganglion cells and fibrous tissue. In workup, they are usually non-functional and detected as a bulky incidentaloma.[2] They are rare, with an estimated incidence of one per million people, and only accounting for 0.2%-2% of all adrenal tumours.[3] They are most often sporadic although can rarely be associated with genetic syndromes (i.e., MEN2 or neurofibromatosis type 1). The diagnosis is rarely suggested pre-operatively given the lack of pathognomonic imaging findings. Ganglioneuromas are frequently

atypical with heterogeneity, contrast washout of <50%, and calcifications.[4] Diagnosis relies on histopathological features. Current evidence supports an excellent prognosis, with a low post-operative recurrence rate.[5]

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Plurihormonal TSH and growth hormone secreting pituitary macroadenoma

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Thyrotropin-secreting pituitary adenomas (TSHomas) are a rare entity, representing approximately 0.5-2% of all pituitary tumours, with an estimated prevalence of around one case per million.[1] They are characterised by autonomous secretion of thyroid-stimulating hormone (TSH), leading to the hallmark finding of elevated free thyroid hormone levels and an inappropriately non-suppressed TSH. Diagnosis requires dynamic testing along with exclusion of interference from laboratory assays or medications.[2]

The case is a 50-year-old male with a background of obesity and osteoarthritis, on no regular medications, who presented to a local hospital in December 2019. He complained of abdominal distension, palpitations, dyspnoea, weight loss of 15kg, increased shoe size, skin tags, and poor libido. He was found to have atrial fibrillation, mild acral features, and clinical features of biventricular heart failure. Initial testing revealed a free thyroxine of 44pmol/L (RR 7.0-17.0pmol/L), a free triiodothyronine of 6.6pmol/L (RR 3.5-6.0pmol/L) and TSH of 6.0mU/L (RR 0.3-4.5mU/L) and an elevated IGF-1 level of 86nmol/L (RR 8.8-29nmol/L). Echocardiogram revealed an ejection fraction of 10-15%. Thyroid interference studies, TRH stimulation test, growth hormone suppression test and magnetic resonance imaging was undertaken to confirm the diagnosis of a TSH/GH co-secreting pituitary macroadenoma. Octreotide long-acting release (LAR) was commenced in May 2020 to achieve a euthyroid state prior to transsphenoidal surgical resection; surgery ultimately was significantly delayed due to the coronavirus pandemic. After two months, he had achieved euthyroidism and IGF-1 was reduced to 39nmol/L, later becoming normalised after 6 months. He underwent surgery in June 2021 with histopathology confirming a plurihormonal Pit-1 lineage adenoma with positive staining for TSH and growth hormone.

A recent review of published TSHoma cases demonstrated approximately 40% of patients had pluri-hormonal tumours, with co-secretion of growth hormone being most common.[3] Therapeutic response to somatostatin analogues is dependent on the relative pattern of somatostatin receptor expression.[4]

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Apparent primary aldosteronism: a case of mistaken identity

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Introduction

Primary aldosteronism (PA) is suspected clinically based on an elevated aldosterone-renin ratio (ARR). However, aldosterone may be overestimated on immunoassays in chronic kidney disease (CKD). We present a case of a young woman with malignant hypertension and end stage renal failure, who had apparent PA on immunoassay but had low true aldosterone levels on liquid chromatography-tandem mass spectrometry.

Case description

A 19-year-old woman was referred for investigation of apparent PA. She had presented with bilateral retinal haemorrhages, hypertension of 180/90 mmHg and new oligo-anuric renal failure with no medical history, medication use or family history. On examination, she weighed 32kg with a height of 146cm, with a BMI of 15 kg/m^2; investigations demonstrated anaemia and secondary hyperparathyroidism, consistent with some chronicity of renal failure. Haemodialysis was initiated, and a renal biopsy showed collapsing focal segmental glomerulosclerosis; evaluation for autoimmune, infective or obstructive causes was negative.

Her ARR was elevated at 332 [<71] using the DiaSorin Liaison XL chemiluminescence immunoassay. A non-dedicated computed tomography scan showed no obvious adrenal pathology. Interestingly, aldosterone decreased despite volume reduction on two sets of pre- and post-dialysis specimens subsequently (see Table 1). After seeking a second opinion, two aldosterone levels of 3 and 2 pmol/L [sitting 0-400, standing 30-800] were measured using liquid chromatography-tandem mass spectrometry; this finally excluded PA as a cause of her presentation.

Discussion

Primary aldosteronism is difficult to diagnose in CKD both clinically and biochemically. The aldosterone-renin ratio is elevated in CKD, but patients may not have spontaneous hypokalaemia. Commercial immunoassays without an extraction step can overestimate aldosterone by up to 50% even in moderate CKD. This is likely due to the accumulation of urinary metabolites such as aldosterone-18-glucoronide and tetrahydroaldosterone-3-glucoronide. Liquid chromatography-tandem mass spectrometry avoids this issue, and should be considered when PA is being considered in CKD.

Date	Aldosterone (100 – 950 pmol/L)	Renin concentration (10 – 50 mIU/L)	ARR (<71)	Potassium (mmol/L)	Medications
Day 9 of admission	663	2	332	4.5	Amlodipine, moxonidine, prazosin
Re-admission	>2770	9	-	4.3	Pre-dialysis
Two months after discharge	2050	10	205	4.4	Post-dialysis Prazosin, moxonidine
Four months after discharge	1050	6	175	4.3	32.6kg pre-dialysis
	538	8	67	2.9	31.4kg post-dialysis Prazosin, moxonidine

Table 1. Aldosterone, renin and aldosterone-renin ratio measurements.S

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Successful parathyroidectomy for primary hyperparathyroidism in the first trimester of pregnancy: case report and review of the literature

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Introduction

Primary hyperparathyroidism in pregnancy is rare, with an estimated prevalence of 1%.[1] Symptoms of hypercalcaemia are often non-specific in pregnancy.[2] At calcium levels >2.86mmol/L, a 3.5-fold increase in pregnancy loss is observed in those with primary hyperparathyroidism.[3] Curative parathyroidectomy is often delayed until the second trimester of pregnancy given the theoretical risk of miscarriage with anaesthesia and surgery.[4] However, hypercalcaemic-related miscarriages often occur during first, or early second trimester of pregnancy; suggesting the need for earlier intervention.[3]

Case Presentation

We describe a case of a 37-year-old woman who underwent a successful parathyroidectomy at six weeks gestation for primary hyperparathyroidism.

M.M. initially presented with anxiety, malaise and a three-week prodrome of constipation and polydipsia, despite consuming 3L of water a day. On examination, she was normotensive with no palpable neck masses or lymphadenopathy. She had a miscarriage four months prior, whereby a corrected calcium was elevated at 3.06mmol/L.

Serial biochemistry was consistent with primary hyperparathyroidism; corrected calcium of 3.16mmol/L, PTH 27.6pmol/L, phosphate 0.75mmol/L, ALP 72U/L and Vitamin D 53nmol/L. Serum Beta-CTx was 1500ng/L and urine calcium:creatinine ratio was 1033mmol/mol (see Table 1).

A 20x6x8mm parathyroid gland was localised on ultrasonography and she underwent a minimally invasive parathyroidectomy two days after her initial presentation. Successful resection of the lesion was demonstrated by an intraoperative PTH fall from 21.4pmol/L to 3.6pmol/L over ten minutes. She experienced no post-operative complications and was discharged 2 days after surgery. She continues to progress well through her pregnancy.

Currently, no guidelines exist for managing primary hyperparathyroidism in pregnancy. Only two cases of parathyroidectomy in first trimester of pregnancy have been described.[5,6] Our case report and literature review add additional insight into the growing body of evidence surrounding the safety of early surgical intervention in severe symptomatic primary hyperparathyroidism to prevent adverse maternal-foetal outcomes.

	Pre-surgery				Post-surgery			Reference Range (non-pregnant)
	3/10/20	27/1/21	2/2/21	4/2/21 Day of Surgery	5/2/21 Day 1 Post-op	8/2/21	8/4/21	
Corrected Ca (mmol/L)	3.06	3.16	3.17	3.08	2.43	2.43	2.36	2.10-2.60
Ionised Ca (mmol/L)		1.87						1.12-1.30
PTH (pmol/L)		27.6	21.4	21.4	2.3	2.8		1.9-8.5
Phosphate (mmol/L)	0.78	0.75	0.69	0.88	1.02	1.09		0.75-1.5
25-OH Vitamin D (nmol/L)		53	90					>50
ALP (U/L)	70	72			60		50	33-96
Beta-CTx (ng/L)		1500						150-800
Albumin (g/L)	42	40	42	33	29	37	33	37-48
Creatinine (nmol/L)	47	50	52	53	57	48		44-80
Urine Ca/Cr ratio (mmol/mol)		1033				693		100-580
Urine Calcium Excretion (umol/L)		52						133-543

Table 1. Biochemistry results of M.M. from diagnosis of her hypercalcaemia to surgery with non-pregnant reference ranges. Rapid normalisation of corrected calcium is seen Day 1 post parathyroidectomy with sustained response two months after.

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Alemtuzumab-induced Graves' disease

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A 22 year old female was diagnosed with Graves' disease (GD) with ocular manifestations eight months following her first dose of alemtuzumab prescribed for refractory relapsing-remitting multiple sclerosis (RRMS). She underwent a total thyroidectomy, and her TSH receptor antibody (TSHrAb) levels remained elevated. She had progression of her ocular disease with marked proptosis and associated exposure keratopathy.

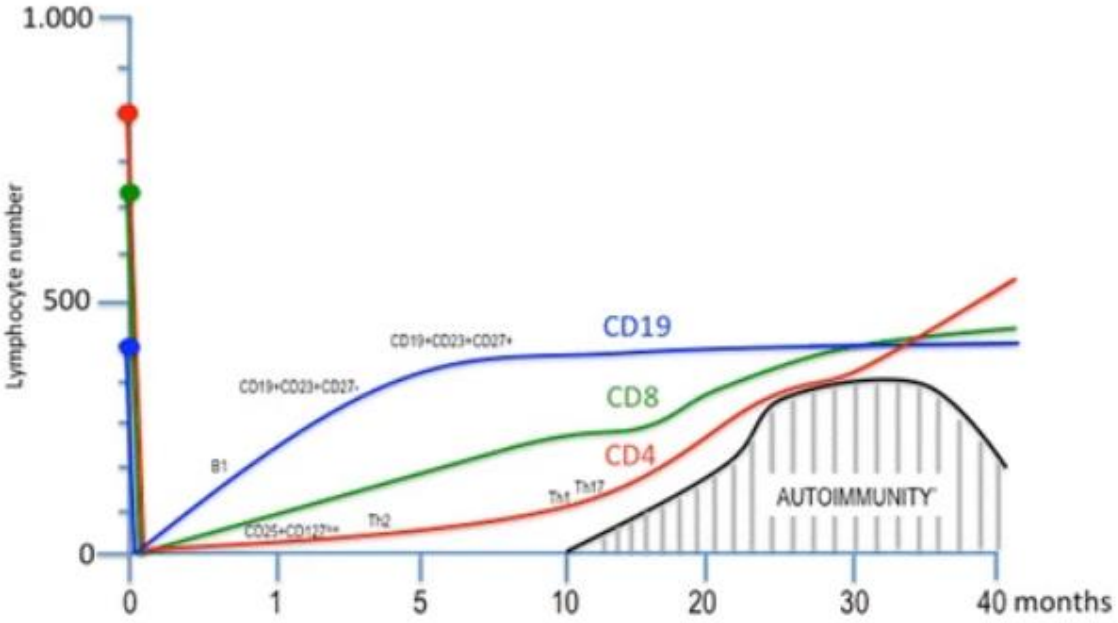
Alemtuzumab is a humanised monoclonal antibody targeting CD52, a transmembrane protein expressed in T and B cells, which reduces relapse rate and disability progression among patients with RRMS.(1-2) Shifts in lymphocyte kinetics lead to undesirable reconstitution autoimmunity.(3-5) The profound lymphopaenia within days of infusion depletes $CD4^+$ T regulatory cells (80%), $CD8^+$ T lymphocytes (>80%) and mature B lymphocytes (>85%). This is followed by repopulation of B-cells, $CD8^+$ T-cells, and $CD4^+$ T-cells in sequence over time (Figure 1).(6-7) The marked hyper-repopulation (180%) of immature B-cells and the slower repopulation of memory $CD4^+$ T-cells and T regulatory cells predisposes to autoimmunity.(6-7)

Autoimmune thyroid disease is the most common reconstitution autoimmune disorder that develops in patients treated with alemtuzumab; up to 70% of patients who develop thyroid dysfunction have GD.(3) Treatment options for alemtuzumab-induced GD include anti-thyroid medications, radioactive iodine therapy and surgery.

The TSHrAb occurs in 30-40% of these patients, and interestingly, TSHrAb in patients with alemtuzumab-induced GD can be stimulatory, inhibitory or neutral, making the disease course difficult to predict.(3) Up to 15% of these patients have a fluctuating course of disease, transitioning from a hyperthyroid to a hypothyroid state and vice versa.(3)

Graves' eye disease is rare; only seventeen cases have been reported in the literature (2 moderate; 6 severe).(5) However, this could be an underestimate as routine ophthalmological assessment was not performed in all patients.

Figure-1. Time-course repopulation of lymphocytes in patients treated with alemtuzumab.



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Plasmapheresis as a bridge to urgent thyroidectomy in a patient with Type 2 Amiodarone Induced Thyrotoxicosis: A case report and review of literature.

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Type 2 amiodarone-induced thyrotoxicosis (AIT2) is a result of destructive thyroiditis and systemic release of thyroid hormones.[1] In AIT2, corticosteroid therapy is recommended, however thionamides and Lugol's iodide are ineffective.[2,3] In those who have contraindications to steroids and/or require rapid reduction in thryoid hormone levels, plasmapheresis may be an option.[3,4] We herein report a case of AIT2 in a 68-year-old man who required rapid and urgent control of his thyroid state to allow

a partial hepatectomy to be safely performed to remove a hepatocellular carcinoma with radiological features suggestive of a high risk of rupture. Corticosteroids were relatively contraindicated in the perioperative setting. Therefore, total thyroidectomy was determined to be the most appropriate and definitive therapy to normalise his thyroid function in anticipation of hepatic resection. To achieve a rapid reduction in circulating thyroid hormones, plasmapheresis was successfully utilised as a bridge to thyroidectomy. A hemi-hepatectomy subsequently followed, without significant complications. Therefore, this case illustrates the utilitisation of plasmapheresis in a patient with severe thyrotoxicosis due to AIT2 preceding thyroidectomy. Features of this case are discussed, together with its implications, and a review of the relevant literature.

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Refractory hypokalaemia - An unusual case of a metastatic neuroendocrine tumour

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A 67-year-old man presented with asymptomatic unexplained hypokalaemia on a background of metastatic ileal neuroendocrine tumour (mNET). It was diagnosed in 2004 and treated with long-acting somatostatin analogue (SSA) and surgical resection. Structural imaging in 2014, performed for increased flushing, found enlargement of hepatic metastatic lesions. Given disease progression, peptide receptor radionucleotide therapy (PRRT) with ^{177}Lutetium Octreotate (LuTate) was commenced with disease control for 6 years.

Routine biochemistry showed unexplained hypokalaemia with metabolic alkalosis. Investigation results were consistent with Ectopic ACTH Cushing's syndrome (Table 1). Pituitary magnetic imaging did not show an adenoma. Biopsy of omental metastasis remained as. a well-differentiated grade 1 NET with immunohistochemistry negative for ACTH. He was urgently treated with Lutate, Metrypone and ketoconazole.

	Result	Normal Range
Serum potassium (Nadir)	2.2 mmol/L	3.5 – 5.2
Spot urine potassium	35 mmol/L	-
24 hour Urine potassium excretion	117 mmol/24hours	50 - 140
24 hour Urine Free Cortisol	6991 nmol/24hours	10 – 150
Renin	< 2.0uIU/mL	4 – 40
Aldosterone	143 pmol/L	30 – 650
ACTH	282 ng/L	7 – 60
Bicarbonate	44 mmol /L	22 – 32

Despite improvement in 24hr Urinary-Free-Cortisol with steroidogenesis inhibitors, he had rapid clinical deterioration. Bilateral adrenalectomy was considered high risk, but deemed urgent and occurred within 2 weeks without immediate complication. Histology showed hyperplasia of the zona glomerulosa consistent with chronic hyperstimulation with ACTH.

Cushing's syndrome is a state of mineralocorticoid excess due to substrate saturation of the 11 beta-hydroxysteroid dehydrogenase leading to more bioactive available cortisol binding to mineralocorticoid receptors and resulting in hypokalaemia [1].

In a series of 166 patients with Cushing's syndrome, 3.6% had a carcinoid tumour causing ectopic ACTH production [2]. Interestingly in the case presented, the metastatic small bowel NET was diagnosed over 15 years before the rapid development of the Cushing's syndrome. Most cases of ectopic ACTH production are synchronous with NET diagnosis (within three months [3]).

Ectopic ACTH syndrome has high mortality. In cases of inoperable disease, that do not respond to medical therapy, bilateral adrenalectomy can definitively cure the Cushing's syndrome. Despite systemic reviews showing high mortality post bilateral adrenalectomy (1 year mortality rate 46% [4]) it is often required to manage refractory disease.

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Adrenal cushing's syndrome with bilateral adrenal lesions: A comparative case study with adrenal vein sampling

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ACTH-independent Cushing's syndrome (CS) is typically caused by autonomous cortisol secretion from a single adrenal adenoma or carcinoma. The diagnosis and subsequent management of such patients can be complicated by the discovery of bilateral adrenal lesions on imaging; each lesion may be a non-functioning incidentaloma or a source of hypercortisolism. In this setting, adrenal vein sampling (AVS) may be utilised to determine lateralization of cortisol secretion. Here we discuss two such patients with contrasting adrenal pathology and post-operative management.

Patient #1 was an active 79-year-old female. Patient #2 was a 70-year-old retired male. Both presented with incidentally discovered adrenal lesions and had Cushingoid features, including hypertension, proximal myopathy and predominantly abdominal weight gain. Each had a suppressed ACTH, low DHEA-sulphate and failed a 1mg dexamethasone suppression test.

Both patients underwent AVS. Patient #1 demonstrated lateralization to the side of the larger adrenal lesion. Patient #2's results did not show lateralization. Successful adrenal vein catheterisation was confirmed in Patient #2 using plasma metanephrine concentrations[1]. Both patients underwent unilateral adrenalectomy, with Patient #2 planned for future contralateral adrenalectomy.

Patient #1 required post-operative glucocorticoid replacement and continues on hydrocortisone while remaining clinically well. Her histopathological diagnosis was adrenocortical adenoma. Patient #2 developed post-operative complications, including intra-abdominal collections and non-occlusive venous thromboses. He was given short-term stress glucocorticoid cover. His histopathological diagnosis was primary bilateral macronodular adrenal hyperplasia; he is planned for future contralateral adrenalectomy.

We conclude that the results of AVS assisted in clinical decision-making in these two patients. We will discuss the role and technique of AVS in patients with adrenal CS and bilateral adrenal lesions. We will also review the post-operative management and follow-up of such patients in the context of the published medical literature.

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Case report: A rare case of hypoglycaemia due to endogenous hyperinsulinism

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Hypoglycaemia is common among hospital inpatients and is most often iatrogenic in nature and mediated by excessive or inappropriate insulin therapy or sulphonylurea use. Hypoglycaemia is a distinctly uncommon entity in people without diabetes. Endogenous hyperinsulinism is a rare but important cause of hyperinsulinaemic hypoglycaemia, which can prove difficult to diagnose and manage.

We describe a case of in a 72 year old man, who initially presented with biventricular cardiac failure and developed recurrent, severe hypoglycaemia, which was insulin-dependent in nature. His medical history was significant for oesophageal adenocarcinoma treated with Ivor-Lewis oesophagectomy 4 years prior. Despite extensive investigations including functional imaging and localisation with a calcium stimulation study, the cause was not identified. Non-insulinoma pancreatogenous hypoglycaemia syndrome (NIPHS) was suspected. The patient experienced progressive, recurrent and severe hypoglycaemia resulting in hypoglycaemic seizures. Medical therapy including somatostatin analogues and diazoxide was ineffective or not tolerated. Ultimately the patient underwent a diagnostic laparoscopy, which proceeded to a distal pancreatectomy. The patient's hypoglycaemia resolved immediately post-operatively. Histopathology of the resected pancreatic tissue revealed diffuse beta-cell hyperplasia consistent with a diagnosis of NIPHS. The patient was cured of his hypoglycaemia and remains well 18 months after surgery.

NIHPS is a very rare disorder characterised by endogenous hyperinsulinaemic hypoglycaemia due to proliferation of pancreatic beta islet cells and is a disease entity distinct from insulinoma, post gastric bypass hypoglycaemia and nesidioblastosis; the latter being a common cause of congenital hyperinsulinism in infants due to mutations in a number of genes governing beta cell function and insulin secretion. Limited case reports suggest adults with NIHPS usually have no prior history of hypoglycaemia, are male

with normal BMI and have a history of non-bypass upper gastrointestinal surgery. The aetiology of the disorder in adults remains unclear and needs further clarification.

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Depot GnRH antagonist for long-term treatment of ovarian hyperthecosis with diagnosis and efficacy established by multi-steroid liquid chromatography-mass spectrometry profiling

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Case Description: A 58-year-old woman presented with severe hyperandrogenism (serum testosterone 15.7-31.0 nmol/L; by LCMS) with menopausal serum LH and FSH, and virilisation but no adrenal or ovarian mass lesions on imaging. Multi-steroid profiling (15 steroids) of adrenal and ovarian vein samples identified strong gradients in left ovarian vein (10-30-fold vs peripheral serum in 17OHP4, 17 OHP5, A_4, T, DHEA), no abnormal adrenal steroid gradients but right ovarian vein could not be cannulated. An opportunistic second left ovarian vein cannulation confirmed an 18-fold gradient in T and > 60-fold gradients in 17OHP4, 17OHP5, A_4 and DHEA. Presumptive diagnosis of OHT was confirmed by a single dose of a pure GnRH antagonist (80 mg Degarelix acetate, Ferring) producing a rapid (< 24 hr) and complete suppression of all ovarian steroids, as well as serum LH and FSH, lasting at least 8 weeks, associated with clinical improvement in virilization. Side-effects include transient injection site reaction and flushing. A second injection was administered at week 8, again with mild injection site reaction and transient vaginal spotting. Serum testosterone remained suppressed at 313 days after the first dose despite recovery of gonadotropins to menopausal levels by day 278 days after first injection. No evidence of any ovarian lesion was reported on surveillance ultrasound.

Conclusions: This case illustrates the diagnosis and long-term treatment of OHT in a postmenopausal woman with severe hyperandrogenism without adrenal or ovarian lesion using multi-steroid LCMS profiling of ovarian and adrenal vein samples. A single dose of a depot pure GnRH antagonist produced rapid and long-term complete suppression of ovarian steroidogenesis for over 10 months. This illustrates the utility of a depot pure GnRH antagonist for rapid confirmation of diagnosis as well as for inducing long-term remission of severe hyperandrogenism from OHT while avoiding pelvic surgery.

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Give steroids! Or don't, it depends: Pitfalls of adrenal insufficiency

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Adrenal insufficiency may be misdiagnosed and inadequately treated in acute situations. A wide spectrum of presenting symptoms can delay recognition and lead to avoidable deterioration. Targeted strategies should be employed for patients with known diagnoses of adrenal failure, ensuring prompt triage and treatment. High-dose parental corticosteroids are the mainstay of treatment in hospital settings. Conversely, injudicious corticosteroid administration and a failure to consider differential diagnoses increases the risk of adverse events.

Two contrasting cases are presented. Case 1: a 21-year-old female with congenital adrenal hyperplasia due to 21-hydroxylase deficiency, presented to a rural Emergency Department with viral symptomology amid the COVID-19 pandemic. Emesis prevented her enteral sick-day action plan and a previous failure of the medical team to recognise the importance of her diagnosis, delayed her presentation for parenteral hydrocortisone. On this occasion, rapid recognition and treatment prevented the impending adrenal crisis, a catastrophic outcome especially in the context of an unknown COVID-19 status. Case 2: a 54-year-old male with known adrenal suppression secondary to long-term prednisolone therapy following renal transplant presented with signs of sepsis, three days after a cholecystectomy. Recurrent episodes of hypotension in the context of presumed ongoing sepsis triggered repeated and extended stress dose corticosteroid and vasopressor treatment, distracting from hypovolaemic circulatory failure. Eventually the heralding signs of melaena and accompanying fall in haemoglobin unveiled gastrointestinal haemorrhage. Eventual recognition triggered appropriate investigations and tailored tapering of corticosteroids by the Endocrinology team.

These cases demonstrate the importance of early recognition and consideration of differential diagnoses in acute presentations. Multi-level approaches are critical: patient empowerment to recognise symptoms and institute their sick-day plan; delivery of parenteral hydrocortisone during ambulance transfer; a system-based approach to identify at-risk patients; robust clinical assessment and appropriate dosing of steroid therapy. Early involvement of the Endocrinology team may reduce excess morbidity and mortality.

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A missed case of TSH producing pituitary macroadenoma

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A 32-year-old woman was noted to be tachycardic during elective surgery. Thyroid function was abnormal and she was referred to endocrine team. She gave 10 year history of galactorrhoea, palpitations , tremor and diaphoresis with frontal headaches. There were no clinical features suggestive of Acromegaly. Heart rate was 100 beats /minute and regular. There was no visual field deficit or clinical features of acromegaly.

Investigation	Initial	Repeat	Reference range
TSH	2.8	1.94	0.5 - 4.5 mIU/L
FT4	20.6	25.8	10-20 pmol/L
FT3	8.8	8.3	3.1-5.4 pmol/L
Prolactin		936	90 - 630 mIU/L
IGF 1		51	9-33 nmol/L
SHBG		83	25-90 nmol/L

Assay interference was excluded as a cause for the thyroid dysfunction. The differential diagnosis was TSH producing pituitary adenoma versus resistance to thyroid hormone (RTH) .She then underwent a TRH stimulation test with results consistent with a diagnosis of a TSH secreting macroadenoma . Post TRH administration, TSH did not show a 2-fold rise, with a pre-TRH level of 2.38 mIU/L and post-TRH level of 2.31 mIU/L at 20 minutes and 1.88 mIU/L at 60 minutes.

Time (min)	TSH (mIU/L)	Free T4 (pmol/L)
Baseline	2.38	38
0	IV bolus of 200 ug of TRH	
20	2.31	4
60	1.88	37

Pituitary MRI showed a large cell mass with suprasellar extension measuring 1.7 x 1.6 x 2 cm approaching optic chiasm. Normal pituitary gland could not be identified as a separate structure.

TSHomas are a rare cause of hyperthyroidism and account for less than 2% of all pituitary adenomas . Most TSH-secreting adenomas secrete only TSH. Approximately 20 to 25 percent of the adenomas secrete predominantly growth hormone or prolactin. Hyperprolactinemia is not always due to tumour secretion of prolactin but can be caused by compression of the pituitary stalk with interruption of hypothalamic inhibition of prolactin secretion.

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Gestational Diabetes Insipidus: The importance of treating the clinical signs and not just the numbers.

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Gestational Diabetes Insipidus (GDI) is a rare complication in pregnancy. It is a transient process secondary to vasopressinase production and release from placental trophoblasts. This may result in decreased circulating arginine vasopressin despite appropriately increased endogenous production from the posterior pituitary gland.

A 31-year-old woman, G4P2, presented at 33 weeks gestation with a five-day history of polydipsia and polyuria. She has a past medical history of migraines; she was taking was pregnancy multivitamins only. Initial evaluation demonstrated a serum sodium 139 mmol/L (135-145), serum osmolality 280 mOsmol/kg (275-295), and urine osmolality 239 mOsmol/kg (300-900). She was normotensive 120/77mmHg, had normal liver transaminases and anterior pituitary hormone levels. Her 24hr urine output was 4.1. Foetal assessment was normal including ultrasonography. A water deprivation test was undertaken with strict safety endpoints. She had near-normal capacity to concentrate urine (endpoint urine osmolality 648 mOsmol/kg, serum sodium 139 mmol/L and serum osmolality 279 mOsmol/kg). She had an excellent clinical response to desmopressin 100mg orally.

Despite inconsistent biochemical markers, her clinical presentation was strongly suggestive of GDI. She discharged home on desmopressin and remained well until 38 weeks plus 2 days of gestation. She represented with worsening polyuria despite desmopressin. Unfortunately, her desmopressin was not titrated due to persistently normal serum sodium levels. She became mildly hypertensive, 132/87mmHg, and had mildly elevated alkaline phosphatase of 134 U/L (30-110). Foetal ultrasonography demonstrated oligohydramnios. She underwent induction of labour (IOL) for reduced foetal movement. Her desmopressin was ceased day of delivery, however, she was re-admitted 48 hours post discharge with ongoing polyuria and clinical dehydration. Recommencement of desmopressin resolved all symptoms. It was ceased eight weeks postpartum and she remains well.

This case report highlights the importance of diagnosing and treating patients with clinical symptoms of GDI despite inconsistent baseline biochemistry to minimise maternal and foetal complications.

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PTH-independent hypercalcaemia due to Pneumocystis jiroveci pneumonia in a renal transplant recipient

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A 68-year-old female presented to hospital with exertional dyspnoea five years post renal transplant for end-stage renal failure secondary to diabetic nephropathy. On admission, she was detected with hypercalcaemia (corrected calcium 3.39mmol/L), elevated phosphate (1.65mmol/L), non-suppressed PTH (7.9pmol/L; reference range 1.7-10.0pmol/L) and a mild acute kidney injury in the context of known previous hyperparathyroidism (PTH of 48pmol/L, corrected calcium 2.64mmol/L and phosphate 1.05mmol/L one month prior). The significant reduction in PTH was suggestive of new PTH-independent hypercalcaemia.

CT chest demonstrated bilateral diffuse ground-glass opacities and pulmonary fibrosis, not suggestive of TB or lymphoma. Due to clinical improvement with empirical ceftriaxone and doxycycline, the patient was planned for discharge as per Respiratory colleagues. However further investigations were performed due to the concern of a systemic process causing PTH-independent hypercalcaemia.

Her serum 25-hydroxyvitamin D level (55nmol/L; reference range >50nmol/L), 1,25-dihyroxyvitamin D level (118pmol/L; reference range 50-190pmol/L) and Angiotensin-Converting Enzyme level were unremarkable and myeloma screen was negative. Sputum samples and later bronchoscopy confirmed Pneumocystis jiroveci pneumonia (PJP), which is known to cause PTH-independent hypercalcaemia. Intravenous pamidronate 30mg was administered to manage persistent hypercalcaemia while investigations were being performed. Corrected calcium improved to 2.66mmol/L. Five months post treatment of PJP, hypercalcaemia resolved (2.50mmol/L) and PTH increased (144.0pmol/L) consistent with longstanding secondary hyperparathyroidism. She remains normocalcaemic two years later.

Cases of hypercalcaemia due to PJP have been previously reported (1-12). A granuloma-mediated mechanism with extrarenal production of 1-alpha hydroxylase has been hypothesised (1). However in our case, macroscopic bronchoscopy was unremarkable, no granulomas were noted on imaging, and serum 1,25-dihyroxyvitamin D level was not elevated, which has been infrequently reported in previous cases (2).

In patients with chronic hyperparathyroidism, hypercalcaemia with a reduction in PTH should prompt consideration of PTH-independent causes. In immunocompromised patients with PTH-independent hypercalcaemia and exertional dyspnoea, PJP should be considered.

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Progressive panhypopituitarism: A case of IgG4-Related Hypophysitis

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Case: A 68-year-old male presented with 6 months of decreasing libido, erectile dysfunction and increase lethargy. Pituitary panel revealed hypogonadotropic hypogonadism with FSH 2.3 IU/L, LH 1.7 IU/L, testosterone 1.2 nmol/L. MRI pituitary showed an abnormally thickened and enhanced pituitary stalk and posterior pituitary measuring 1cm x 0.6cm and extending up to the hypothalamus. Pan CT revealed mild mediastinal lymphadenopathy. Mediastinoscopy and lung biopsy demonstrated minor chronic inflammatory changes consistent with chronic bronchitis without evidence of malignancy or granulomatous disease. Subsequent PET scan was negative for any abnormal uptake.

Trial of prednisolone 25mg for 3 months resulted in radiological improvement however treatment was ceased due to insomnia. Several weeks later diabetes insipidus and hypothyroidism developed requiring desmopressin and thyroxine replacement.

Pituitary stalk biopsy demonstrated significant fibrous tissue with mixed chronic inflammatory cell infiltrate, that was predominately lymphocytic with a moderate number of IgG4+ plasma cells on immunohistological staining. Serum IgG4 was elevated at 3.32 g/L [reference range 0.030-2.010 g/L]. Hence, a diagnosis of IgG4-related hypophysitis was made, and the patient was recommenced on prednisolone 50mg daily with plan to continue for 3 months before tapering.

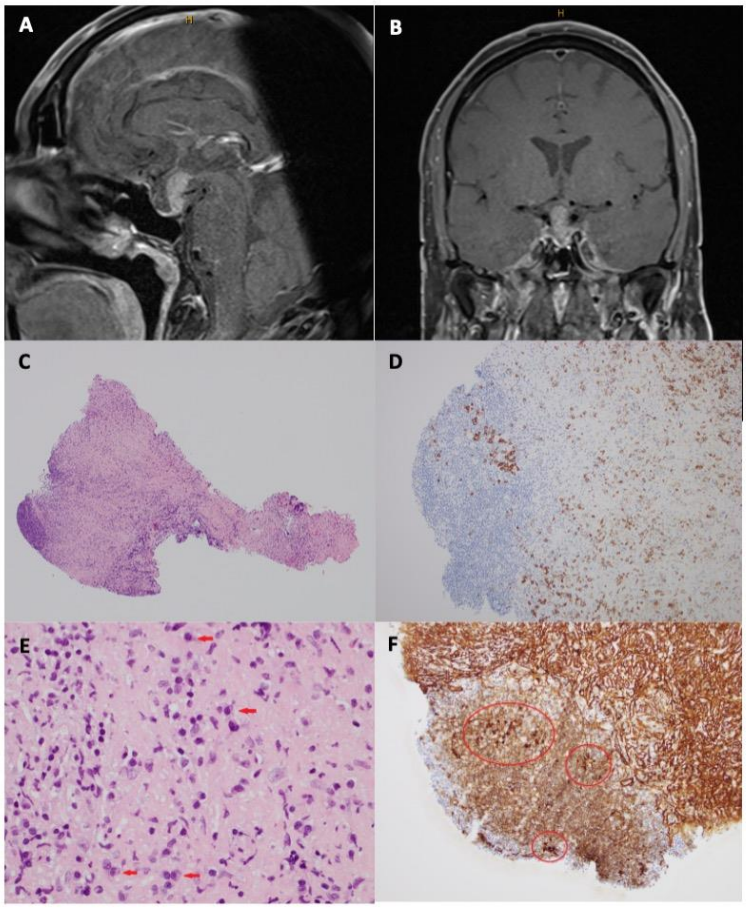

Figure 1: A) & B) showing T1 weighted sagittal and coronal images of MRI pituitary demonstrating an abnormally enhanced and thicken pituitary stalk and posterior pituitary. A) The pituitary fossa is enlarged and filled with CSF. There is loss of the posterior pituitary bright spot and the anterior pituitary is reduced to a layer of tissue on the fossa floor. C-F: Histopathology of pituitary stalk biopsy. C) H&E Stain at low power (x4) demonstrated fibrosis tissue with chronic inflammation. D) Immunohistochemical staining for CD138 (x10) highlighting presence of numerous plasma cells otherwise not appreciated on H&E staining. E) H&E staining (x40) with plasma cells (arrows). F) IgG4 immunohistochemical staining with patches of IgG4+ plasma cells highlighted in red circles. IgG4+ plasma cells to total plasma IgG+ cell ratio unable to be determined due to significant background staining. Ratio of >40% of IgG4+ plasma : IgG plasma cells is diagnostic for IgG4-related disease in any organ.

Discussion: IgG4-related hypophysitis is rare fibroinflammatory disease characterised by dense lymphoplasmacytic infiltrate rich in IgG4+ plasma cells. This condition affects more males than females, often presents in the 6^{th}-7^{th} decade of life with varying degrees of hypopituitarism and/or diabetes insipidus. IgG4-related disease often involve other organs.

Diagnosis is made based on the following criteria:

1. Histopathological findings on pituitary biopsy *or*
2. MRI pituitary features (bulky stella mass with thickened pituitary stalk) plus histological evidence of extra-pituitary IgG4 related disease *or*
3. MRI pituitary features plus elevate serum IgG4 and evidence of steroid responsiveness

Glucocorticoid is first line therapy for IgG4-related hypophysitis. Early treatment may reduce pituitary mass but may not reverse existing endocrinopathy.

Posterior pituitary metastasis from breast carcinoma: a rare presentation in the absence of diabetes insipidus

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Pituitary metastases are rare and often asymptomatic. They usually present with diabetes insipidus.

We present a case of a 64 year old female with a posterior pituitary metastasis and thickening of the pituitary stalk. She had a history of early oestrogen receptor positive, progesterone receptor positive, HER2 negative breast cancer diagnosed 6 years prior. She had been treated with wide local excision, four cycles of docetaxel and cyclophosphamide chemotherapy, adjuvant radiotherapy and five years of exemestane. One month prior to diagnosis of the pituitary metastasis, she had presented with bone metastases to T8, T9, T10 vertebrae, the left acetabulum and bilateral pubic rami.

Magnetic resonance imaging (MRI) demonstrated a thickened pituitary infundibulum with a possible lesion in the anterior pituitary, as well as numerous calvarial and skull base metastases. Serial MRI scans three and four months later demonstrated an increase in size of the stalk measuring 8.5 x 16 mm, with a definitive posterior pituitary lesion. There was superior displacement of the chiasm with new T2 hyperintense signal change of the right optic tract. Repeated visual field testing showed no visual field defects. Her only endocrine dysfunction was hyperprolactinaemia, likely from stalk compression, with a peak level of 1710 mIU/L and low gonadotropins (LH <0.5 IU/L, FSH 11.5 IU/L). She received dexamethasone to reduce oedema, with good effect. At no point did she develop signs or symptoms of diabetes insipidus. A pituitary biopsy confirmed metastatic carcinoma of the breast with the same morphology and hormone profile to the initial breast pathology. She received radiotherapy and is current clinically stable.

Breast cancer is the most common primary malignancy associated with pituitary metastases. The absence of typical symptoms and biochemical changes associated with diabetes insipidus does not exclude this rare, but important, diagnosis.

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An unusual association: papillary and follicular thyroid carcinoma with Graves' thyrotoxicosis and orbitopathy

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Graves' disease has been associated with papillary and follicular thyroid carcinoma, but rarely both carcinomas concurrently. We present a case of papillary and follicular thyroid carcinoma complicated by the additional issue of severe Graves' orbitopathy requiring steroid treatment and surgical management.

A 51-year-old Caucasian man was diagnosed with Graves' hyperthyroidism with associated severe orbitopathy. He had no prior medical history and was not taking any other medication. He had a 15 pack year smoking history, with recent cessation. Thyroid receptor antibody titre was elevated at 35.5 IU/L (< 1.8) and nuclear medicine thyroid scan showed diffusely increased uptake (4.3%). He was treated with propylthiouracil followed by carbimazole, as well as methylprednisolone and selenium for orbitopathy.

In the context of worsening liver function derangement and orbitopathy, thyroidectomy was arranged. Anatomical pathology revealed a multifocal papillary thyroid carcinoma with widespread lymphovascular invasion, with metastatic papillary carcinoma in two of five perithyroidal lymph nodes. The largest focus was 20 mm and tumour was seen less than 0.1 mm from margins. There was also minimally invasive and angioinvasive follicular carcinoma. American Joint Committee on Cancer (AJCC) Staging was pT1b, pN1a.

Subsequently the patient was diagnosed with hepatitis C and treated with glecaprevir and pibrentasvir.

Three months after thyroidectomy, and following right orbital decompression, high ablative dose (4.95GBq) radioactive iodine (I-131) was administered, with enhanced steroid cover in light of his orbitopathy.

This case demonstrates the rare co-occurrence of Graves' disease and papillary and follicular thyroid carcinoma, and highlights successful use of glucocorticoids to reduce the risk of exacerbating Graves' orbitopathy with high dose I-131.

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A series of insulinomas

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We present three cases of newly diagnosed insulinoma at our centre in 2020. AN, a 59yo male, presented with 24 months of intermittent confusion. 72-hour fast confirmed endogenous hyperinsulinaemia, and endoscopic ultrasound and DOTATATE-PET demonstrated a 9mm uncinate process lesion, consistent with an insulinoma. AN underwent Whipple's surgery, had an uncomplicated recovery, and remains symptom free.

DS, a multi-comorbid 88yo female, was referred with acute dysphasia and hemiparesis, with a glucose level of 2.1mmol. 72-hour fast was positive, and CT pancreas suggested a pancreatic tail insulinoma. DS was medically treated with diazoxide, however dose escalation was limited by fluid retention. Glucocorticoids were commenced, and DS remains stable.

PM, a 74yo male, was referred post syncope with a “LOW” blood glucose, on a background of 2 years of similar episodes. Again, 72 hour fast confirmed endogenous hyperinsulinaemia, and imaging suggested a pancreatic tail lesion. PM underwent distal pancreatectomy, and histopathology revealed a 35mm well differentiated neuroendocrine tumour, with metastatic tumour in 1/1 lymph node.

Insulinomas are rare, with an incidence of 4/1,000,000 per year. 10% are malignant, 10% are ectopic, and 10% are associated with an underlying genetic syndrome. Localisation is vital for treatment planning and prognostication. Structural imaging, with MRI or CT pancreas, can be insufficient, particularly in lesions <10mm. Somatostatin receptor expression in insulinomas is variable, and thus DOTATATE PET imaging can be insensitive. GLP1 receptors (GLP1-R) are highly expressed in insulinomas, and GLP1-R PET imaging presents a novel way of identifying lesions. Malignant insulinomas are larger (>25mm), secrete more insulin, and are almost invariably intra-pancreatic. Treatment is generally debulking surgery, even in metastatic disease. Newer management options include mTOR inhibitors, pasireotide and PRRT. Genetic screening should be considered patients who are young, have hyperparathyroidism, or have recurrent disease.

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Is this MEN4? A novel *CDKN1B* mutation in a patient with a clinical diagnosis of MEN1

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MK, 32yo F, presented with primary hyperparathyroidism (PHPT) age 26. Imaging revealed three enlarged parathyroid glands, and she underwent bilateral neck exploration and subtotal parathyroidectomy. There was no family history of endocrinopathy.

Genetic testing was negative for *MEN1* gene mutations, but a *CDKNB1* unclassified gene variant was noted (CDKN1Bc.482c>G p.SER161Cys). Genetic testing revealed her father had the same *CDKNB1* variant, but with no known disease. Given her clinical syndrome, she was commenced on a screening protocol for MEN1.

Pituitary MRI demonstrated a 15x13x12mm left pituitary macroadenoma. Serial MRI abdomen and gastrointestinal hormone testing are normal. Serum calcium has remained between 2.55-2.65mmol/L, with an elevated PTH.

This case demonstrates a patient with clinical evidence of a MEN1 syndrome, manifesting with a pituitary adenoma and parathyroid hyperplasia, with no *MEN1* gene mutation, but a mutation in *CDKNB1* of uncertain significance.

Discussion

5-25% of patients with clinical MEN1 lack typical *MEN1* gene mutations, and are labelled “phenocopies”[1]. Initially described in mouse models, mutations in the *cyclin dependent kinase (CDK) inhibitor 1b* gene (*CDKN1B*) have been identified in patients with multiple endocrine neoplasias, and these patients have been reclassified as MEN4[2].

Identifying pathogenicity of a genetic mutation is challenging. In our patient, the gnomAD database was reviewed and found that 48 healthy patients were heterozygotes for the same mutation as our patient without known manifestations of MEN. The *CDKN1B* gene is known to tolerate mutational burden without impaired gene function. Additionally, our patient’s mutation has not been reported in the literature, and has thus been labelled ‘unclassified’. Nevertheless, MK has a clinical diagnosis of MEN1.

PHPT in MEN1 requires subtotal or total parathyroidectomy. Recurrent/persistent PHPT occurs in 30-66% of patients after 8 years follow up[3]. Ectopic parathyroid glands may be present in thymic tissue, therefore prophylactic thymectomy should be considered with initial surgery[4].

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Hypercalcaemia – a basic case in point

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A 62-year-old man presented with increasing confusion, falls, dysarthria and ataxia. Past medical history included a traumatic brain injury many years ago, with subsequent cognitive impairment and seizures. He resided in a residential care facility.

Initial pathology demonstrated moderate hypercalcaemia of 3.34 mmol/L (2.21-2.63) with an acute kidney injury (eGFR 40 mL/min). There were not thought to be any medications contributing to hypercalcaemia, though it was noted that he was taking colecalciferol 50mcg daily.

Fluid resuscitation was followed by administration of pamidronate 60mg. Subsequent investigations included a low-normal parathyroid hormone level 20 pg/mL (15-68) paired with calcium 2.97 mmol/L, vitamin D 89 nmol/L (>50), 1,25-dihydroxyvitamin D 42 pmol/L (50-190). Screening for solid organ and haematological malignancy was unremarkable. Computed tomography (CT) of the neck/chest/abdomen/pelvis identified a filling defect within the caecum, however a colonoscopy performed with good preparation within one month prior to admission had not demonstrated any abnormality at this site. At the time of discharge, calcium had normalised to 2.47 mmol/L and renal function had partially recovered to eGFR 60 mL/min.

He represented within two weeks of discharge with recurrence of the previous symptoms. Biochemistry was similar to the first presentation. Venous blood gas revealed a pH of 7.39 (7.35-7.45), bicarbonate 42 mmol/L (21-28) and pCO2 69 mmHg (32-48), consistent with a compensated metabolic alkalosis. A careful history identified the frequent ingestion of six to 12 QuickEze tablets per day, each containing calcium carbonate 750mg. Calcium normalised to 2.27 mmol/L with fluid repletion and eGFR recovered to 60 mL/min.

This case highlights the milk-alkali syndrome as an uncommon and under-recognised cause of PTH-independent hypercalcaemia. A high index of suspicion and careful history is needed to elicit potential contributing substances and avoid unnecessary investigations and treatments, while a high bicarbonate level may be useful clue suggesting metabolic alkalosis.

Investigations	**Results**		
Pathology	**First admission**	**Second admission**	**Reference range**
Corrected calcium	3.43	3.73	2.21-2.63 mmol/L
Phosphate	1.04	1.39	0.87-1.45 mmol/L
Parathyroid hormone (PTH)	20	13	15-68 pg/mL
-- Paired corrected calcium	2.97	3.73	2.21-2.63 mmol/L
Vitamin D	89		>50 nmol/L
1,25-dihydroxy vitamin D	42		50-190 pmol/L
Venous pH	7.42	7.39	7.35-7.45
Venous bicarbonate	37	42	21-28 mmol/L
Venous pCO2	57	69	32-48 mmHg
Creatinine	157	159	64-104 micromol/L
eGFR	40	40	>90 mL/min
Chloride	98	95	98-109 mmol/L
Thyroid stimulating hormone (TSH)	1.79		0.35-4.94 mU/mL
Angiotensin converting enzyme (ACE)	40		20-70 U/L
Serum protein electrophoresis (SPEP)	No monoclonal protein		
Kappa light chains	30.1		3.3-19.4 mg/L
Lambda light chains	34.9		5.7-26.3 mg/L
K/L ratio	0.86		0.26-1.65
Radiology			
Renal tract USS	Echogenic foci through the renal medulla, most in keeping with nephrocalcinosis		
Computed tomography (CT) neck/chest/abdomen/pelvis	Ill-defined 3.2cm filling defect within the caecum concerning for polyp, previously demonstrating FDG-avidity in October 2020.		
99m-Tc whole body bone scan	No osteoblastic metastatic disease		

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A case of pseudohypercalcaemia secondary to paraproteinaemia

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There are rare case reports of pseudohypercalcaemia secondary to calcium-binding paraproteins both from IgM and IgG. We present a case of a 76 year old male with asymptomatic PTH-dependent hypercalcaemia on a background of previous primary hyperparathyroidism treated with 3 gland parathyroidectomy 20 years prior, multiple myeloma under surveillance and bladder cancer awaiting resection.

Laboratory data showed corrected calcium 2.91mmol/L, PTH 3.6pmol/L, Vitamin D 80nmol/L, 24 urine calcium 3.1mmol/24hr with a FeCa - 0.0093 and phosphate 1.6mmol/L. PTHrp was normal at <1.0pmol/L and CASR testing showed no variant of clinical significance. Serum electrophoresis demonstrated elevated IgG and Kappa ratio. Bone marrow aspiration showed plasma cell myeloma with low level marrow infiltration (15%). BMD demonstrated a lowest T score of -1.1 at the femoral neck, bone scan did not reveal any metastatic bone disease and he had no history of renal calculi. Ionised calcium was at the lower limit of the normal range, and on retrospective review was below the reference range prior to his parathyroidectomy, raising the suspicion for pseudohypercalcaemia in the setting of paraproteinemia.

Our patient had long-standing MGUS prior to diagnosis of multiple myeloma and his Kappa IgM paraproteins were confirmed to be negatively charged, therefore plausibly falsely elevating serum total calcium levels. Further laboratory investigations are pending to confirm pseudohypercalcaemia secondary to paraproteinaemia. This case highlights the importance of excluding pseudohypercalcaemia prior to initiating treatment; particularly in the setting of paraproteinaemia and we suggest monitoring ionised calcium rather than corrected calcium.

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Hormonal Heartbreak

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A 70-year-old female with a recent history of anxiety and completely resolved Takotsubo cardiomyopathy, presented with chest pain and mild hypertension (SBP 154mmHG) associated with anterior ST changes and troponin of 2052ng/L (RR <16). Coronary angiography was normal and transthoracic echocardiogram demonstrated an EF of 27%. She was treated with oral metoprolol for a presumed recurrent Takotsubo's cardiomyopathy.

Within 24 hours she developed acute cardiogenic shock with bradycardia requiring ICU support and potential transfer for extracorporeal membrane oxygenation. A CT to investigate aortic dissection was negative but a 6cm right adrenal mass was identified.

Endocrine were consulted and plasma metanephrines, cortisol and ACTH, and an aldosterone/renin ratio were sent. Due to high clinical suspicion for pheochromocytoma, patient was commenced on alpha blockade with oral prazosin and all beta-blockers were strictly withheld.

All plasma metanephrines were significantly elevated; metadrenaline 36,000pmol/L (RR 30-540), normetadrenaline 20,000pmol/L (RR 13-1600) and 3 methoxy Tyramine 682pmol/L (RR<120).

Cortisol and ACTH were also elevated at 1649nmol/L (RR 80-480nmol/L) and 15.7pmol/L (RR <14) which raised suspicion of an ACTH-producing phaeochromocytoma. A 1mg overnight dexamethasone suppression test was also abnormal, with post-dexamethasone cortisol 970nmol/L. Clinically the patient was not cushingoid.

Two weeks after the acute presentation, her cardiac function improved to an EF of 55%. ACTH and cortisol also normalised (3.2pmol/L and 279nmol/L respectively), suggesting the significant hypercortisolaemia represented an acute stress response.

Four weeks after the initial presentation, she underwent a laparoscopic right adrenalectomy. Tumour cells stained positive for synaptophysin and did not have cytological features of malignancy with total PASS score of zero. Total tumour weight was 84.1g. Post-operative echocardiogram demonstrated reduction in EF to 45%, suggesting effects of intraoperative catecholamine release, despite alpha and beta blockade.

This case demonstrates the importance of high clinical suspicion for pheochromocytomas or paragangliomas in Takotsubo or hypertension induced cardiomyopathy.

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Adrenal gland haemorrhage following motor vehicle accident with resultant adrenal insufficiency

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We present two cases of traumatic adrenal haemorrhage resulting in hypocortisolism, with different presentations and outcomes, highlighting the need to consider adrenal failure in trauma patients.

Case 1: A 60-year-old man presented following a high-speed motor vehicle accident (MVA). Initial trauma CT demonstrated hepatic and bilateral adrenal haemorrhages. He had significant haemodynamic instability and was commenced on intravenous hydrocortisone. The 8AM cortisol on day six, after withholding hydrocortisone the prior afternoon, was 57 nmol/L (100-540 nmol/L)

with an associated rise in noradrenaline requirements. Over the next ten days, hydrocortisone was weaned to an oral replacement dose. Two weeks later, after withholding the afternoon hydrocortisone, the 8 AM cortisol was 533 nmol/L with ACTH 34 pg/ml (<46 pg/ml) and hydrocortisone was ceased. Follow-up CT scan four months later demonstrated resolution of the adrenal haemorrhages, but a persisting 20 x 22 mm low density mass (5-13 Hounsfield units (HU)), within the left adrenal gland consistent with an adenoma.

Case 2: An 88-year-old woman on dabigatran for atrial fibrillation presented following a high-speed MVA, sustaining multiple fractures, a left-sided subdural haematoma and a subcapsular splenic haematoma. Initial trauma CT did not demonstrate any adrenal injury. She was haemodynamically stable throughout her week-long admission and was discharged to rehabilitation. Three days later, she became significantly hypotensive and was readmitted for vasopressor support. Repeat abdominal CT demonstrated new bilateral adrenal haemorrhages. Serum cortisol was <28 nmol/L and she was commenced on intravenous hydrocortisone. Persisting primary adrenal insufficiency was confirmed one week later, with 8 AM cortisol 129 nmol/L and ACTH 82 ng/L. Intravenous hydrocortisone was weaned to an oral replacement dose. Repeat CT scan four months later demonstrated complete resolution of the adrenal haemorrhages. However, she has persisting adrenal insufficiency, with 8 AM cortisol 43 nmol/L and ACTH 66.2 pmol/L and remains on hydrocortisone replacement.

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A dopamine-secreting subcarinal paraganglioma

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A 74-year-old man was referred for investigation of an incidentally found subcarinal paraganglioma on the background of worsening cardiac function.

His past medical history included a jugulotympanic paraganglioma resected in 1997 with complete remission.

He presented with dyspnoea. CT imaging noted a pulmonary embolus and a 2.8cm lesion initially thought to be subcarinal lymph node. DOTATATE-PET demonstrated intense subcarinal node uptake, and moderate uptake at the previous site of jugulotympanic paraganglioma resection (Figure 1).

Histology of the subcarinal lesion demonstrated a likely paraganglioma with a Ki67 of <2%. He had no clinical features of a phaeochromocytoma. Plasma normetadrenaline and noradrenaline were normal but Plasma 3-methoxytyramine (3-MT) was elevated (Table 1). Given his cardiac failure, and unknown growth rate, a decision was made for monitoring. Four months later, he was re-admitted to hospital due to a symptomatic decline in his LVEF (48 to 30%) on serial TTEs (Table 2). Cardiac failure was optimized with beta blockade.

Repeat CT and PET scans revealed no interval growth 9 months later, but his LVEF deteriorated to 24% despite medical optimization

Table 1: Plasma catecholamines

	Normetanephrines (<900 pmol/L)	Metanephrines (<500 pmol/L)	3-methoxytramine (<110 µg/L)
Admission for workup of declining LVEF	547	431	288
6/10/20 (During admission)	551	384	323
8/10/20 (During admission)	346	244	315
7/6/21 Repeat testing as outpatient	776	375	169

Table 2: Echocardiogram findings

Date	Left ventricular ejection fraction	LV filling pressure
20/02/2020	48%	Normal
05/10/2020	30%	Normal
21/01/2021	35-40%	Elevated
15/06/2021	34%	Elevated, E'e= 13

FDG PET (27/08/20)

DOTATATE-PET (24/9/20)

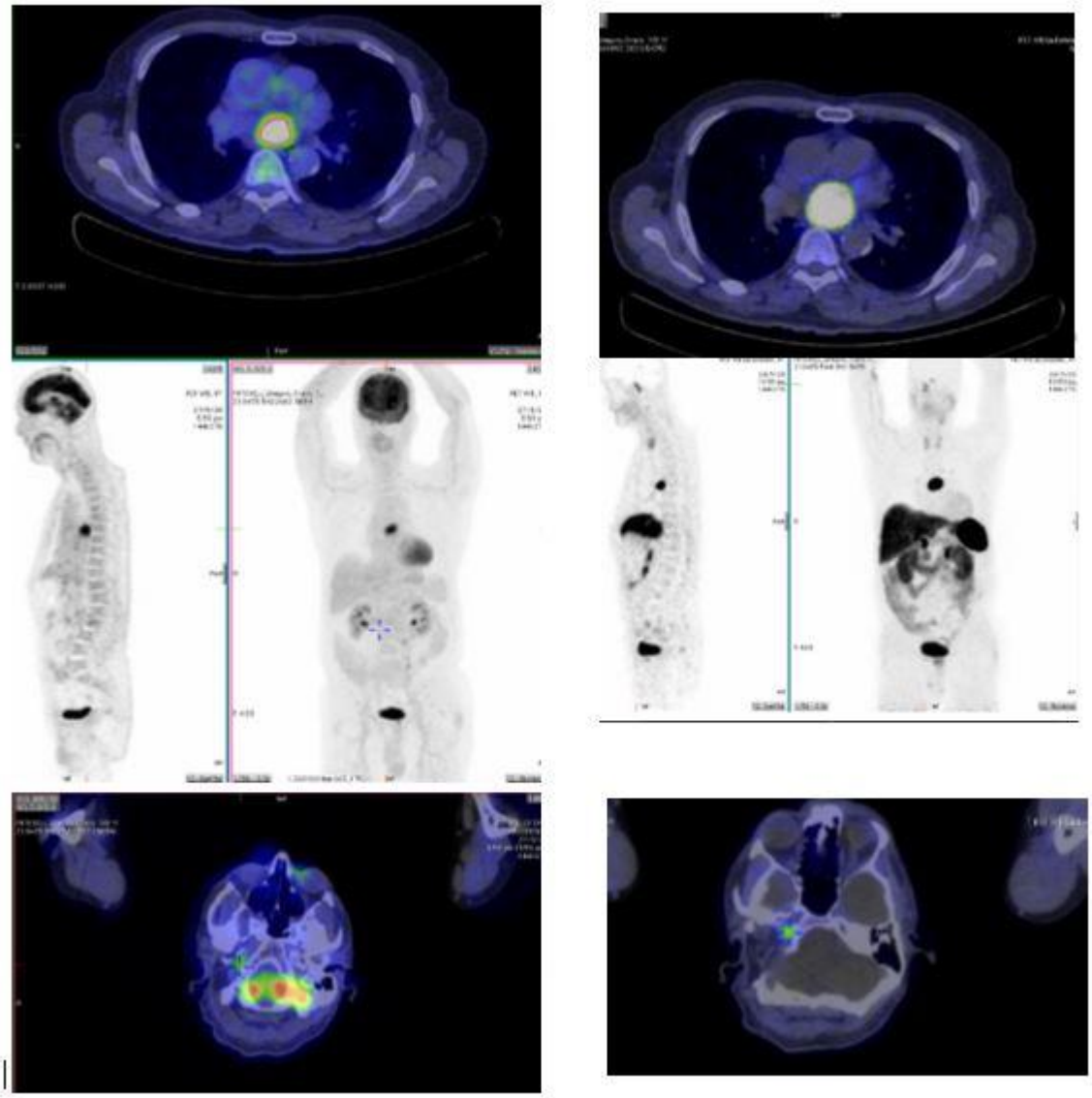

Figure 1: FDG- and DOTATATE-PET findings.

Discussion

Paragangliomas that exclusively produce dopamine are very rare and limited to mostly single case reports. Compared to (nor-)metadrenaline secreting phaeochromocytoma/paraganglioma the are characterized by a lack of typical paroxysmal features. The predominance of dopamine and lack of production of other catecholamines is due to deficiency of dopamine β-hydroxylase. This is due to de-differentiation of the tumour and thus explains the higher malignancy potential.

One of the unique aspects of this case is whether the dopamine excess contributed to his progressive cardiac failure. However, there have been no documented cases of heart failure associated with dopamine-producing paragangliomas. At low doses, dopamine preferentially binds the Dopamine-1 and Dopamine-2 receptors causing vasodilatation, hence alpha blockade is not indicated as can worsen hypotension via further vasodilatation.

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Primary hypophysitis with syndrome of inappropriate ADH and rhabdomyolysis in a previously well man

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A 45 year-old man presented after a fall with a 2-minute lie, one week of bifrontal headaches, myalgias and subjective fevers and was found to have profound hypo-osmolar hyponatraemia (serum sodium 104mmol/L). He had been prescribed Zopiclone and

Panadeine forte one week prior. On examination, he was euvolaemic. There were no abnormal neurological signs apart from slowed mentation.

Biochemistry revealed an elevated urine osmolality (589mOsm/Kg) and sodium (65mmol/L) consistent with SIADH. A pituitary panel revealed hypogonadotropic hypogonadism and mild central hypothyroidism (Table 1). Creatine kinase was profoundly elevated (39,171 U/L). A rheumatological screen was unremarkable. A pituitary MRI revealed a slightly bulky pituitary gland with homogenous enhancement suggestive of lymphocytic hypophysitis (Figure 1).

His sodium incremented gradually with a 3% saline infusion and 1L fluid restriction and eventually normalised one week after discharge. His myalgias and CK resolved spontaneously. His thyroid and gonadal axis also normalised spontaneously two weeks later. A pituitary MRI five weeks later showed a normal pituitary gland.

Table 1 Pituitary panel on admission, and several days later

	7/4/21	10/4/21	13/4/21
TSH (0.27-4.20 mU/L)	0.10	1.33	1.61
T3 (3.3-6.8pmol/L)	3.6	3.6	4.5
T4 (8.00-16.50 pmol/L)	6.2	9.3	12.6
ACTH (7.2-63.3ng/L)	46.9		58.4
Cortisol (185-624nmol/L)	778		459
GH (<0.97 ug/L)	<0.05		<0.05
IGF-1 (9.62-29.51 nmol/L)	25.04		22.3
FSH (1.3-19.3 IU/L)	5.0		8.5
LH (1.3-8.6 IU/L)	0.7		4.4
Testosterone (9.0-28.3IU/L)	0.6		4.9

ACTH= adrenocorticotrophic hormone, GH= growth hormone, IGF-1= insulin-like growth factor1, FSH= follicle stimulating hormone, LH= luteinizing hormone

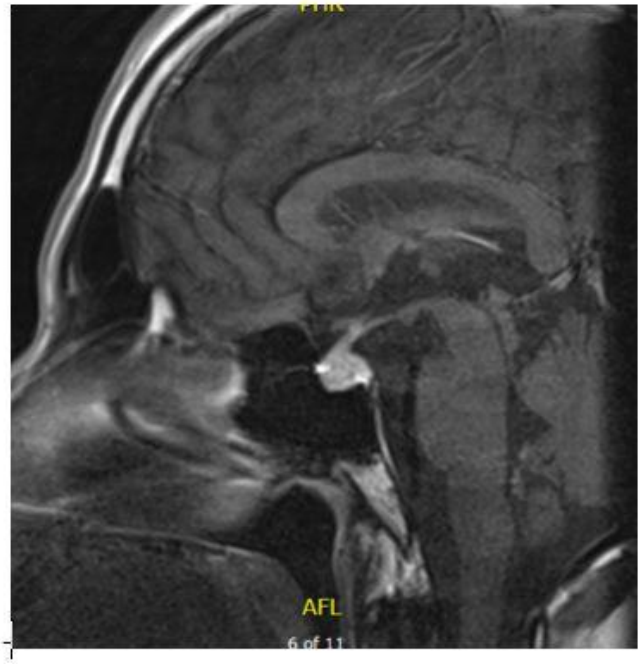

Figure 1: MRI pituitary demonstrating Mildly enlarged pituitary gland with thickened but midline pituitary stalk suggestive of lymphocytic hypophysitis

Table 2: Neuroradiological features of autoimmune hypophysitis vs pituitary adenomas.

	Autoimmune hypophysitis	**Adenoma**
Symmetrical	Yes	No, sprouts towards cavernous sinus
Homogenous	Yes	No, cystic/necrotic areas
Gadolinium uptake	High	Lower (lower vascular attenuations)
Thickened stalk	Typical (also present in lymphoma, sarcoidosis, histiocytosis)	Rare
Posterior bright spot	Frequently lost	Usually conserved
Association with pregnancy	Yes	No
Pituitary volume	Smaller	Larger (>6cm^3)

Table 3: Summary of retrospective cohort studies of treatment of primary hypophysitis

Study		Observation	Glucocorticoids	Surgery
Honeggar, 2015	Radiological regression	45% (10/22)	65% (19/29)	42% (12/28; no recurrence)
	Endocrine improvement	27% (6/22)	15% (4/26)	8% (2/25)
	Endocrine improvement	15% (5/33)	100% (4/4)	25% (1/4)
Wang, 2017	Radiological regression	N/A	52.9±16.7%	57.3±20.3%
	Endocrine improvement	0% (0/5	41% (9/22)	0% 0/5
Oguz, 2019	Radiological regression		4/4	
	Endocrine improvement	N/A	25% 1/4	
Kyriacou, 2017	Endocrine improvement		0% 0/12 at 5 yrs	1/5 at 5 yrs
Panigrahi, 2018	Radiological regression	N/A	80% (4/5)	100% (4/4)
	Endocrine improvement		20% (1/5)	0% 0/4

Lymphocytic hypophysitis is rare. The most common presenting symptoms include headache, visual disturbance and symptoms resulting from loss of pituitary hormonal function.

Suggestive features on MRI to distinguish hypophysitis from adenomas include an enlarged pituitary with thickened stalk (Table 2).

The management of hypophysitis involves hormonal replacement of the affected axis. High dose glucocorticoids have been trialled in patients with severe symptoms (Table 3). Surgery has been performed in cases with neurological symptoms or headache unresponsive to glucocorticoids.

SIADH has not previously been described in patients with primary hypophysitis. Other factors that could contribute to this included the use of opiates, and presence of pain.

Rhabdomyolysis has only been described in those with hypophysitis with concomitant cortisol deficiency. While there have been case reports of hyponatraemia as a cause of rhabdomyolysis, this has not been replicated in animal models.

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Non-islet cell tumour hypoglycaemia: a rare but serious paraneoplastic syndrome

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A 79-year-old male presented with a reduced level of consciousness and hypoglycaemia with a background of metastatic sarcoma from a right lower lobe solitary fibrous tumour. Baseline blood tests revealed mildly raised inflammatory markers with normal thyroid function and cortisol level, no source of infection was localised. He responded well to prednisolone and 50% dextrose infusion, with complete resolution of neurological symptoms. Further investigation whilst hypoglycaemic revealed low insulin, c-peptide and beta-hydroxybutyrate in keeping with the diagnostic criteria for IGF-2 mediated hypoglycaemia, a subtype of non-islet cell tumour hypoglycaemia (NICTH).(1)

A rare but sinister complication of malignancy, NICTH is caused by tumour secretion of insulin-like growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2) or glucagon like peptide (GLP 1).(2) As in the case of our patient with a presumed diagnosis of IGF-2 mediated hypoglycaemia, mesenchymal tumours are most commonly associated.(3) IGF-2 induces hypoglycaemia through multiple actions. Gluconeogenesis, glycogenolysis, ketogenesis, lipolysis and activity of glucose 6 phosphatase are all inhibited. Additionally, IGF-2 increases glucose demands by muscles.(4) These metabolic pathways are stimulated by IGF-2 as the amino acid polypeptide shares 47% sequence homology with insulin.(5)

Clinically, patients with paraneoplastic production of IGF-2 generally present with neuroglycopaenic symptoms.(6) Biochemical evaluation at the time of hypoglycaemia reveal decreased levels of insulin, proinsulin, C-peptide and beta-hydroxybutyrate.(1) IGF-1 and IGF-2 levels can be measured, typically resulting in a raised IGF-2 to IGF-1 ratio.(4) In the case of our patient, IGF-2 could not be tested as no Australian lab performs this assay. Following initial correction of hypoglycaemia, optimal long term therapy is surgical resection of the causative tumour which can result in cure. Medical therapy may be used for symptom relief when targeted treatment of malignancy is not feasible. This includes glucocorticoid, recombinant human growth hormone (rhGH) and glucagon use.(7)

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A case series and literature review of Necrobiosis lipoidica diabeticorum

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Necrobiosis lipoidica diabeticorum (NLD) is a rare, chronic disease characterised by clinical features of yellow-brown, atrophic, telangiectatic plaques usually located on the lower extremities, and pathological features of collagen necrobiosis and inflammation in the dermis. Most cases are seen in patients with diabetes mellitus, particularly type 1 diabetes, and in patients without diabetes most have evidence of abnormal glucose tolerance or report a family history of autoimmune disease . We describe three patients with NLD and type 1 diabetes: (1) a 24-year-old female with extensive erythematous plaques responsive to laser therapy, (2) a 24-year-old female with recalcitrant localised disease despite prolonged treatment with multiple therapeutic agents including topical and systemic steroids, calcineurin inhibitors, and hydroxychloroquine, and (3) a 22-year-old female who reported longstanding brown-to-red papules localised on the shins, with biopsy demonstrating classical features of NLD; she is yet to start treatment. A common theme to all three cases is the late identification and delay in reaching the correct diagnosis. Hence, we discuss the important clinical characteristics and features and discuss the management and prognostic implications for this distinctive cutaneous entity. Whilst most cases will remain relatively asymptomatic, others progress to a more debilitating illness with pruritus, dysesthesia, and pain. Pain is often intense in the presence of ulcerated plaques, the latter being an unfortunately morbid complication of NLD. The clinicopathological diagnosis of NLD requires the integration of both the clinical and histopathological findings. NLD has proved a challenging condition to treat, and despite the numerous therapeutic modalities available, there remains no established standard regimen of care. We provide an overview of current management strategies available for NLD.

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Y am I Male

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Introduction:

Isodicentric Y Chromosome (IDYC) is the most common chromosomal structural anomaly and may not be diagnosed until adulthood. IDYC presents with complications that need monitoring and management by endocrinologists.

Case:

A 28-year-old male was referred for low libido on a background of Hashimoto's thyroiditis. Examination revealed short stature (150cm), Tanner stage 5 secondary sexual characteristics, a right testis measuring 18ml with an absent left testis and hypospadias. Sex Hormone profile was consistent with primary hypogonadism. Semen Analysis revealed azoospermia. Peripheral Karyotyping showed mosaicism with (90%)45,XO and (10%)46,X,isodicentric Y. FISH study located a breakpoint and fusion at 11.2222 on the Y chromosome. Computed Tomography revealed a left intra-abdominal gonad, which was removed. On Histology, the presumed undescended testis was a Mullerian structure consisting of a fallopian tube and rudimentary uterus. Karyotyping of this showed mosaic IDYC anomalies.

Discussion:

Male sex differentiation is determined by the SRY gene and hormones including AMH and Testosterone[1]. Disruption of this leads to disorders of sex development.

Breakage and deletion along the Y chromosome and fusion of sister chromatids, leads to the formation of IDYC[2]. Phenotypic manifestations are determined by mosaicism and what genetically important components of the Y chromosome are deleted [3]. Common phenotypes include Turner syndrome, infertility, gonadal dysgenesis and short stature.

IDYC accounts for 10-15% of male infertility[4]. If oligospermia is present, testicular sperm extraction can be performed. For those not wanting fertility, testosterone replacement improves outcomes[5]. The risk of testicular cancer in dysgenetic gonads is increased and requires annual ultrasonography surveillance[6].70% have a short stature due to the deletion of the SHOX gene[4]. The benefit of Growth Hormone replacement for this cohort is conflicted.

Conclusion:

Management of IDYC must be individualised given the heterogeneous phenotypes. This includes assessing gonadal malignancy risk, hormonal replacement and assisted reproductive techniques.

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A long awaited diagnosis

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We present the case of MR AB, a 30-year-old male, referred in August 2020 with central hypogonadism and a 10-year history of polydipsia and polyuria with an overnight water-deprivation test consistent with diabetes insipidus. His remaining pituitary profile was unaffected, and he was found to have pituitary stalk abnormalities on MRI. Following limited biochemical and radiological response to a steroid-course, he underwent a pituitary-stalk biopsy with histopathology consistent with IgG4 related hypophysitis (IgG4-RH). At present, no extra-pituitary manifestations of IgG4 related disease has been demonstrated, and he remains clinically well on GnRH analogue and desmopressin therapy.

IgG4-RH is diagnosed upon meeting criteria as described Leporati et al.(1). Given increasing recognition of its poorly specific nature, the need for a set of diagnostic criteria that can better differentiate between true IgG4-RH vs other pituitary autoimmune and inflammatory entities (which also meet Leporati's biopsy criteria) has arisen (2,3).

In this clinical case study, we will review the strengths and pitfalls of our current diagnostic pathway for the diagnosis of IgG4-RH. We will describe the emergency of two distinct clinical phenotypes of IgG4-RH, which, while sharing many biochemical, radiological and histopathological findings, may either represent two distinct pathologies or part of a spectrum of the same disease process.

TAKE HOME POINTS

- IgG4 RH is a relatively new entity, likely underdiagnosed, and can present with a normal serum IgG4 level.

- With prompt identification and treatment with glucocorticoid/immunosuppressive therapy there is generally an excellent response to treatment
- While biopsy has historically been the gold standard for diagnosis, there are cases of non-IgG4 related disease fulfilling criteria for IgG4-RH.
- Similarly, when diagnosing IgG4-RD based on an abnormal MRI appearance and elevated serum IgG4, one must keep in mind alternate causes for these changes

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The benefit of leftovers following bilateral adrenalectomy for Cushing's disease

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Background: Following bilateral adrenalectomy (BLA) serum cortisol is typically undetectable. Though in 12% endogenous cortisol production may recur in the form of adrenal remnant and rest tissue. We present a patient with Cushing's disease (CD) managed with BLA and pituitary radiation with no glucocorticoid replacement for over 10 years, before presenting with an adrenal crisis.

Case Presentation: A 54 year-old male presented acutely unwell with fatigue and the sensation of felling hot. His blood pressure was 99/83mmHg, pulse 112/min and he had an altered level of consciousness. On physical examination he had bilateral laparotomy scars, and initially had hyponatremia. A diagnosis of adrenal crisis in the setting of sepsis was made and he was treated with intravenous fluids, intravenous hydrocortisone and antibiotics, with improvement in his clinical state within 24 hours.

He was diagnosed with CD at age 20 and at the time was managed with BLA and prophylactic pituitary radiation. Post-BLA he was replaced with hydrocortisone and fludrocortisone, however he self-ceased it more than a decade ago and was lost to follow-up. Despite not been on glucocorticoids for over 10 years, he reported been well, having reasonable energy levels and had not required any hospital admissions.

Incidentally during his admission, abdominal CT scan showed residual adrenal tissue on the right-side. His morning cortisol prior to hydrocortisone was 211 nmol/L, and the rest of his pituitary profile is enclosed in table 1. Given his adrenal crisis and BLA, he was discharged home on hydrocortisone 20mg in the morning and 10mg at midday, in addition with 50mcg of fludrocortisone.

Conclusion: Following BLA for Cushing's disease, endogenous cortisol production may recur. Therefore clinicians should regularly review the need and dosage of glucocorticoids, and if Cushingoid features reappear further evaluation should be pursued looking for clinically significant adrenal remnant and rest tissue.

Table 1: Pituitary profile during admission

	Result	Reference Range
Cortisol at 10:50am	211	08:00am 140 – 640 nmol/L
ACTH	35	10 – 50 ng/L
T4	12	7.0 – 17 pmol/L
TSH	4.5	0.3 – 4.5 mU/L
LH	4.3	1.0 – 9.0 U/L
FSH	3.5	1.0 – 15 U/L
Testosterone at 10:00am	3.3	9.0 – 35 nmol/L
SHBG	29	10 – 50 nmol/L
IGF-1	5.6	8.1 – 28 nmol/L

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A case of metastatic adrenocortical carcinoma with sustained clinical response following immunotherapy and review of current treatment options

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Background

Adrenocortical carcinoma is a rare malignancy of the adrenal cortex with an estimated annual incidence of one to two per million population per year. Patients often present with rapid disease progression. The current mainstay of treatment for metastatic disease is with mitotane plus combination chemotherapy; however, mortality remains high and further options are needed.

Case

A 74-year-old-woman was incidentally noted to have an adrenal lesion measuring 30x21x30mm with a non-contrast density of 30HU in early 2018 upon investigation of shortness of breath. A repeat CT abdomen/pelvis performed 4 months later showed doubling of the adrenal lesion size to 56mm with a density of 40HU. Hormonal testing was unremarkable. She underwent surgical resection with histopathology confirming a 90mm adrenocortical carcinoma with extracapsular extension and vascular invasion but no involvement of the removed 15 lymph nodes. Microscopy reported aggressive features including high mitotic activity with ki67 index of 40% and negative staining for DNA mismatch repair proteins MSH2 and MSH6. She was treated with mitotane therapy for 2 months but an FDG-PET scan demonstrated multiple new glucose avid abdominal lymph nodes and pulmonary metastases with the largest lesion measuring 20mm in the right middle lobe. She was administered a single dose of ipilimumab and nivolumab after which she developed immune-related hepatitis. On serial imaging she demonstrated impressive response with reduction in size of the pulmonary lesions and has subsequently remained clinically and radiologically stable after 3 years.

Discussion

There have been clinical trials assessing the use of immune checkpoint inhibitors with varying results. The key predictors of response appear to be tumour factors including microsatellite instability, PDL-1 positivity and mismatch repair abnormalities. Therapies such as IGF1 receptor inhibitors, VEGF inhibitors and EGFR inhibitors have provided disappointing results to date. This case help illustrate the emergence of newer, more effective treatment options.

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Metastatic papillary thyroid carcinoma in pregnancy

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Thyroid disease is common in pregnancy but malignant thyroid nodules are rare. We present a pregnant woman diagnosed with differentiated papillary thyroid cancer (PTC) which progressed to metastatic disease.

A 28 year old presented at 15 weeks gestation for management of subclinical hypothyroidism with a TSH of 4.8 and free T4 of 8.2. An incidental non-tender nodule was noted. Thyroid ultrasound conveyed a 6mm x 8mm x 10mm TI-RADS 5 right lower pole solid nodule with internal microcalcification. Subsequent FNA indicated PTC Bethesda 6 classification. MDT discussion with patient and family concluded delaying surgery until postpartum was appropriate. She underwent serial sonography second monthly. Postnatally, a CT neck showed no lymphadenopathy though noted concerning pulmonary nodules. Consequently, she underwent a total thyroidectomy two months postpartum. Histopathology confirmed a 12mm right PTC with two separate 8mm foci of micropapillary thyroid carcinoma and two of six metastatic lymph nodes. She was referred for radioactive iodine.

Thyroid cancer in pregnancy is challenging. The mainstay in evaluation is ultrasonography with TI-RADS classification. FNA of suspicious nodules is safe in pregnancy with use of Bethesda classification to determine further management. Pregnancy termination is not required for PTC. Definitive treatment involves subtotal or complete thyroidectomy but surgery timing during pregnancy has various complexities. Without surgery, there is potentially an increased risk of progression due to hormonal effect, though this remains unsubstantiated. Surgery carries a higher chance of complications including mortality and longer hospital stay. Consensus is to avoid surgery in first and third trimesters due to increased risk of foetal and maternal complications.

Given the delay in surgical management, early CT radiography was and should be considered post-delivery to detect metastatic disease. Although uncommon, the risk of progression should be discussed with women and their families to ensure an informed decision is made regarding treatment options.

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Assessment of adrenocortical dysfunction in the context of hypoalbuminaemia: a case report and review of the literature

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Introduction:

Individuals with cortisol deficiency have an excess mortality when faced with acute stressors.[1, 2] However, there is no consensus on the assessment of adrenocortical dysfunction in individuals with hypoalbuminaemia and presumably low cortisol-binding globulin (CBG) levels.

Case presentation:

A 62 year old man was admitted with recurrent fevers and confusion. He was malnourished and had multiple opportunistic infections in the context of receiving a liver transplant 14 months prior, with good graft function. He was hypotensive, and the 0730am serum cortisol was low at 161 nmol/L (RR 170-500), serum ACTH was 19.1 pmol/L (RR ≤10), and serum albumin was 17 g/L (RR 33–48). Plasma renin was 607 fmol/L/sec (RR 130-2350), aldosterone was 186 pmol/L (RR 60-980), and the aldosterone/renin ratio was low at 0.2 (RR 0.4-1.5). A Short Synacthen Test (SST) was abnormal (baseline serum cortisol 149 nmol/L, peak cortisol 249 nmol/L at 60 mins). There was no history of long-term glucocorticoid therapy. Adrenal gland CT did not

demonstrate any abnormality. He was receiving long-term fluconazole treatment for pulmonary cryptococcosis. He was commenced on hydrocortisone and fludrocortisone therapy, and responded well clinically.

Discussion:

Approximately 70–80% of circulating cortisol is bound to CBG, 10-20% is albumin-bound, and the remainder (<10%) is available as free cortisol.[3-7] A large randomised trial suggested that hydrocortisone and fludrocortisone therapy was associated with reduced risk of death in critically-ill patients with 'relative adrenal insufficiency' (determined using SST).[8] Another study reported that ~40% of critically-ill patients with hypoproteinaemia had lower baseline and cosyntropin-stimulated serum total cortisol concentrations, but similar baseline and cosyntropin-stimulated serum free cortisol concentrations, compared with those with near-normal albumin concentrations.[9] This presentation will review the evidence regarding the interpretation of serum cortisol levels in individuals with hypoalbuminaemia, and the utility of assessing serum free cortisol and salivary cortisol measurements.

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Thymic carcinoid in a patient with multiple endocrine neoplasia type 1

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Case: A 54-year-old man underwent a partial resection for a thymic carcinoid in 2008 followed by radiotherapy. He was subsequently found to carry the *MEN1* gene mutation. His other manifestations of MEN 1 included primary hyperparathyroidism, a growth hormone-secreting pituitary adenoma, neuroendocrine tumours of the pancreas and liver and a gastrinoma. Due to ongoing tumour progression, he was treated with capecitabine and temozolamide in 2019. In 2020, he presented with worsening dysphagia and superior vena cava obstruction due to local tumour compression. He was admitted to hospital and treated with dexamethasone and palliative radiotherapy to the mediastinum. He experienced hypoglycaemia associated with high C-peptide, insulin and proinsulin levels, raising the suspicion of a proinsulin-secreting insulinoma. This was initially treated with cornstarch and diazoxide, but the diazoxide was switched to octreotide due to oedema. In line with his wishes for second-line palliative chemotherapy, he was commenced on carboplatin and etoposide. Although he tolerated his second cycle of chemotherapy poorly, necessitating an admission to the palliative care unit for presumed end-of-life care, he made a remarkable recovery, and repeat imaging has demonstrated stable disease nine months later.

Discussion: We present the case of a complex patient with thymic carcinoid occurring in the context of many concurrent manifestations of MEN 1. This case illustrates the difficulties of treating thymic carcinoid given its aggressive nature. There is a limited evidence base to guide therapeutic options, which include surgical resection, radiotherapy and adjuvant chemotherapy. More investigational treatments include somatostatin analogues, peptide receptor radionuclide therapy and everolimus. However, despite this and multiple other manifestations of MEN 1, this patient has demonstrated meaningful progression-free survival.

SRB POSTER ABSTRACTS

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The effect of discrete wavelengths of visible light on the developing murine embryo

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Non-invasive optical imaging has potential in determining developmental potential of the embryo. Such approaches use light at varying wavelengths. The impact of irradiating preimplantation embryos with discrete wavelengths of light has not been investigated appropriately. Light has several parameters that may affect embryos. These include wavelength, the average and peak power, exposure duration, and overall energy dose delivered to the embryo. Our study distinguishes itself from previous work by: (i) ensuring that light is applied uniformly across the embryo; (ii) having accurate calibration of the energy dose; and (iii) a knowledge of the spectral bandwidth of each light source. In this study we performed rigorous comparison between wavelengths by accounting for the above-mentioned factors.

We exposed embryos to blue (470nm), green (520nm), yellow (590nm), or red (620nm) light at varying developmental stages assessing development to blastocyst and, DNA damage, inner cell mass (ICM) and total cell numbers (TCN) in the blastocyst-stage embryo. Four experimental exposure groups were used (unexposed, or from the morula – blastocyst, 4-cell – blastocyst or 1-cell – blastocyst stages), thus, embryos were exposed once a day for 0, 2, 3 or 5 days of preimplantation development.

Compared to unexposed embryos, blastocyst rate was significantly lower in embryos exposed to yellow light from the 1-cell to blastocyst stage ($P<0.05$) and 4-cell to blastocyst stage ($P<0.01$). Significantly higher levels of DNA damage in the blastocyst occurred for most wavelengths and developmental stages exposed, compared to unexposed embryos ($P<0.05$). When compared to unexposed, TCN was significantly lower in red exposed embryos ($P<0.05$). The ICM was not affected by any wavelength.

Our results show that embryo development is impacted not only by wavelength, but also the frequency of exposure and developmental stage exposed, demonstrating that developmental rate alone may not indicate the full impact of light on the developing embryo.

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Characterising novel growth factor receptors in the spermatogonial stem cell population

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Spermatogonial stem cells (SSCs) hold potential to be used as a therapeutic tool to reverse chemotherapy-induced infertility. Unfortunately, *in vitro* culture techniques that are a precursor to these therapies are not robust, with studies showing a 13-fold reduction in SSC number and a 16-fold reduction in regenerative capacity over 6 months[1]. To address this deficiency, this project aimed to discover novel growth factors that can promote long-term self-renewal of the SSC population *in vitro*, through the identification and characterisation of unique membrane receptors.

To identify growth factor receptors that are enriched in self-renewing SSCs, as opposed to downstream progenitor or differentiating spermatogonia, mining of bulk[2]- and single cell-RNAseq databases (unpublished and [3]) was conducted. Platelet-derived growth factor receptor A (PDGFRA) was selected for further investigation due to a 2.77-fold enrichment in transcript levels in SSCs above progenitor spermatogonia ($P<0.00005$) in the postnatal day 6 testis. Further, transcripts for associated ligands (PDGF-A/B) were found to be expressed in several somatic and germ cell populations, suggesting that this growth factor-receptor interaction occurs in the niche *in vivo,* but is not necessarily replicated within current *in vitro* culture conditions. To validate receptor expression at the protein level, a novel transgenic reporter mouse line was used (*Id4-eGFP* mouse[2]) that can delineate SSC and progenitor populations by way of GFP intensity. Using immunocytochemistry, we confirmed that PDGFRA was expressed in 50% of the SSC population, a significant enrichment above progenitor spermatogonia ($P<0.05$). Interestingly, further investigation of ligand expression using immunohistochemistry suggested that PDGF-A production may occur within SSCs themselves, potentially suggesting an autocrine, rather than paracrine, regulatory mechanism. Future experiments will explore the consequences of PDGFRA knockdown on SSC self-renewal capacity. By characterising growth factor-receptor interactions that sustain SSCs, we may be able to adapt *in vitro* culture conditions to better maintain these cells long-term.

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Can we make SpermFAST? Improving sperm function to increase fertilisation rates after *in vitro* fertilisation.

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Intracytoplasmic sperm injection (ICSI) usage (~64% of autologous cycles) has increased in Australia as an attempt to avoid low fertilisation and total fertilisation failure after standard *in vitro* fertilization (IVF). ICSI and to a lesser extent IVF, bypass key sperm maturation (hyperactivation, capacitation/acrosome reaction) events that naturally occur in the female tract and are vital for successful fertilisation. Current commercial sperm preparation media are not fully designed to induce these changes in sperm prior to insemination. The aim of this study was to improve fertilisation rates following IVF by increasing sperm capacitation and hyperactivation between sperm preparation and insemination ultilising a new sperm medium (SpermFAST).

Sperm from 12 consenting normospermic men were incubated in either G-IVF+ (Vitrolife) or SpermFAST (UoA/Monash IVF) following a direct swim-up. Measures of capacitation, hyperactivation, sperm binding, acrosome reaction and oxidative DNA damage were assessed. Further, sperm from male CBAF1 mice (N=8) were incubated in either G-IVF+ or SpermFAST prior to IVF insemination. Fertilisation rates, embryo development, blastocyst cell numbers and DNA damage were assessed.

Incubation of human sperm in SpermFAST increased tyrosine phosphorylation (15.8% vs 9.5%, $P<0.05$), hyperactive motility (38.3% vs 14.9%, $P<0.01$), sperm binding (73.1% vs 47.7%, $P<0.01$), while having no impact on sperm acrosomal status or oxidative DNA damage levels. Following IVF in the mouse, sperm incubated in SpermFAST increased fertilisation rates (94% vs 88%, $P<0.05$), blastocyst total cell (92.2% vs 77.4%, $P<0.05$), inner cell mass (14.9% vs 18.9%, $P<0.01$) and epiblast cell numbers (3.7% vs 1.6%, $P<0.01$), while the proportion of DNA damaged cells decreased in blastocysts (2.3% vs 4.8%, $P<0.001$).

Sperm function and fertilisation rates are improved when the sperm medium better mimics the environment of the female reproductive tract during natural conception. Improving IVF culture media to better meet the physiological needs of sperm could potentially improve outcomes following IVF.

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The effect of in vitro culturing environment on the regulation of histone acetylation in the preimplantation embryo

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Background:

The transition of chromatin from a transcriptionally repressive to permissive state is necessary for the onset of gene expression in the embryonic genome. Acetylation of histone 3 lysine 9 (H3K9ace) is involved in this process. H3K9ace is undetectable in oocytes and embryos that have just been fertilised. However, levels rise throughout early embryonic development and during the start of DNA replication. Interventions carried out during an *in vitro* fertilisation (IVF) process can impact this reprogramming.

Aim:

To investigate factors that may affect the amount of H3K9ace during early embryo development.

Methods:

Using quantitative indirect immunofluorescence, we measured the amount of H3K9ace in zygotes cultured *in vitro* in single step or sequential media (optimal and suboptimal). Additionally, IVF embryos, and embryos obtained after natural mating with and without fixing the culturing and the insemination time were tested.

Results:

In embryos obtained after natural mating H3K9ace expression was significantly greater in single step media compared to sequential media, irrespective of which media was used (optimal or suboptimal). However, using suboptimal sequential media increased the level of H3K9ace. Embryos obtained after IVF demonstrated a higher H3K9ace level compared to embryos obtained after natural mating. On the other hand, when the culture time between IVF and embryos obtained after natural mating was the same, we found an opposite effect. Additionally, narrowing the insemination period resulted in higher levels of H3K9ace in natural mating embryos.

Conclusion:

Our findings demonstrate that *in vitro* culture conditions induce disturbances in the epigenetic reprogramming and the impact is exacerbated when suboptimal media is used. This demonstrates that *in vitro* culture is the main adverse stressor on H3K9ace irrespective of the method of fertilisation, and affirms the usefulness of H3K9ace measurements as a biomarker of stress in the early embryo.

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Defining the niche for vaginal epithelial stem cells.

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Publish consent withheld

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The ketogenic diet may impair embryonic developmental programming via beta-hydroxybutyrate mediated metabolic and epigenetic aberrations

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Background:

Maternal dietary modifications alter the nutrient composition of the preimplantation embryonic environment. This can stimulate persistent metabolic and epigenetic adaptations in the embryo that program development, ultimately affecting child and adult health. The ketogenic diet (KD), which is increasingly popular amongst sub-fertile women trying to conceive, induces elevated maternal ketone levels, including beta-hydroxybutyrate (ßOHB). ßOHB has a known ability to modulate metabolic and epigenetic regulation, however, the impact of elevated ßOHB exposure on embryonic viability and developmental programming remains unknown.

Aim:

To assess the impact of ßOHB on preimplantation mouse embryo development, metabolism, epigenetic state, and post-transfer viability.

Method:

Preimplantation mouse embryos were cultured *in vitro* with or without 2 mM ßOHB, representing serum concentrations with KD consumption. Day 5 blastocyst cell number and lineage allocation was assessed via differential nuclear stain, metabolism of ßOHB and carbohydrates was assessed by ultramicrofluorescence, and acetylation of histone 3 lysine 9 (H3K9ac) and lysine 27 (H3K27ac) were assessed by immunofluorescence. Day 4 blastocysts were transferred to pseudo-pregnant females for analysis of embryonic day 14.5 placental and fetal development.

Results:

A reduction in total and trophectoderm cell number ($P<0.05$) was observed following 96 h exposure to 2 mM ßOHB, indicating reduced viability. Blastocysts were shown to consume ßOHB *in vitro*, with uptake increasing with ßOHB concentration. Further, ßOHB increased blastocyst glycolytic rate ($P<0.01$), while epigenetic analyses revealed H3K9ac and H3K27ac were unaffected. Of significance, post-transfer implantation rates were reduced ($P<0.05$), concurrent with smaller placental diameter ($P<0.01$) and fetal crown-rump length ($P<0.05$), indicating alterations in developmental programming.

Conclusion:

Preimplantation ßOHB exposure induces negative developmental programming effects that are mediated by metabolic aberrations, and plausibly incorporate epigenetic components other than H3K9ac or H3K27ac. There may therefore be negative impacts on the long-term viability and health of offspring exposed to a maternal KD during early pregnancy.

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Analysis of upstream regulators, networks and pathways associated with the expression patterns of polycystic ovary syndrome candidate genes during fetal ovary development

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Polycystic Ovary Syndrome (PCOS) is a multifactorial syndrome with reproductive, endocrine and metabolic symptoms, affecting about 10% women of reproductive age. Pathogenesis of the syndrome is poorly understood with genetic and fetal origins being

the focus of the conundrum. Genetic predisposition of PCOS has been confirmed by candidate gene studies and Genome-Wide Association Studies (GWAS). Recently, the expression of PCOS candidate genes across gestation has been studied in human and bovine fetal ovaries. The current study sought to identify potential upstream regulators and mechanisms associated with PCOS candidate genes. Using RNA sequencing data of bovine fetal ovaries (62-276 days, n = 19), expression of PCOS candidate genes across gestation was analysed using Partek Flow. A supervised heatmap of the expression data of all 24,889 genes across gestation was generated. Most of the PCOS genes fell into one of four clusters according to their expression patterns. Some genes correlated negatively (early genes; *C8H9orf3, TOX3, FBN3, GATA4, HMGA2* and *DENND1A*) and others positively (late genes; *FDFT1*, *LHCGR*, *AMH*, *FSHR, ZBTB16* and *PLGRKT*) with gestational age. Pathways associated with all the genes in each cluster were determined using Ingenuity Pathway Analysis software (IPA), KEGG pathway analysis and Gene Ontology (GO) databases using DAVID bioinformatics. Genes in the early gene group were involved in mitochondrial function, the upstream regulators included *PTEN, ESRRG/A* and *MYC* and oxidative phosphorylation and mitochondrial dysfunction pathways were the top canonical pathways. Genes in the late group were involved in stromal expansion, cholesterol biosynthesis and steroidogenesis and upstream regulators included *TGFB1/2/3, TNF, ERBB2/3, VEGF, INSIG1, POR* and *IL25*. These findings provide insight into ovarian development of relevance to the origins of PCOS, and suggest that multiple aetiological pathways might exist for the development of PCOS.

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RNA-sequencing demonstrates the effects of seminal fluid on uterine transcriptome at implantation and identifies γ/δT cells as the top regulated immune cell population in mice

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Seminal fluid induces major changes in the uterine transcriptome immediately after mating, inducing cytokine and immune cell changes that initiate maternal immune tolerance for pregnancy. However, whether and how seminal fluid contact at mating affects endometrial gene expression subsequently in the peri-implantation phase is unclear. To address this knowledge gap, we utilised high-throughput RNA-sequencing to identify genes and pathways regulated by seminal fluid components in the endometrium of C57Bl/6 female mice on day (d) 3.5 post-coitum (pc) after mating with intact (INT), vasectomised (VAS), seminal-vesicle-deficient (SVX), or SVX/VAS BALB/c males (n=3-4/group). RNA-sequencing and Ingenuity Pathway Analysis revealed that compared to SVX/VAS-mated females, the T Cell Receptor (TCR) Signalling Pathway was the top pathway activated in INT-mated females (Z-score=3.2). Interestingly, γ/δ TCR genes *Tcrg-C1* and *Trdc* (2.8- and 2.2-fold increase), and genes associated with γ/δT cell function including *Il7r* and *Blk* (2.6- and 3.1-fold increase), were amongst the top differentially regulated genes. These changes were mainly driven by seminal plasma, as mating with VAS males induced a similar increase. The γ/δT cell population in the uterus was then assessed using flow cytometry on d3.5pc, with virgin females as estrous control (n=11-12/group). The γ/δT cell population in the uterus was expanded after INT or VAS mating by 8.3-and 10-fold compared to virgin females, with 22.4- and 21.9-fold more γ/δT cells expressing the proliferation marker Ki67. This increase was not evident in females mated with SVX or SVX/VAS males. Together, this study demonstrates that seminal fluid contact alters the endometrial transcriptome at implantation, and identifies expansion of a resident γ/δT cell population as a dominant element of this response. This increase in γ/δT cell number in response to semen has potential to modulate receptivity to embryo implantation, but future studies are required to define its exact contribution to reproductive success.

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Progesterone receptor regulates ovarian granulosa cell transcription during ovulation through interaction with RUNX1 and chromatin remodelling action

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The underlying mechanism of ovulation is of great interest for the development of safer non-hormonal female contraceptives. Ovulation is triggered by the luteinising hormone (LH) surge and a key determinant for ovulation is the LH-induced transcription factor progesterone receptor (PGR). This study sought to identify the effect of the LH surge and PGR on genomic reprogramming in granulosa cells in order to reveal critical mechanisms specific to ovulation. RNA-seq and ATAC-seq of mouse granulosa cells identified large-scale shift in global chromatin accessibility and transcription profile in response to LH-stimulus. Differential analysis of transcription factor binding motifs at open chromatin sites identified two distinct suites of transcription factors that were active before and after the LH-stimulus. Importantly, the canonical binding motif for PGR and other NR3C steroid receptors (GR, AR, MR) was more enriched in regions with reduced chromatin accessibility after LH, whereas motifs for RUNX1 and other transcription factor families were highly enriched among chromatin regions with LH-induced accessibility. This supports our previous demonstration of tissue-specific PGR action through non-canonical motifs, in which ChIP-seq showed that PGR preferrably binds non-canonical motifs corresponding to other transcription factor families in a tissue-specific manner. Comparative ChIP-seq analysis revealed overwhelming mutual chromatin binding between PGR and RUNX1 in granulosa cells, especially at ovulatory gene promoters. Proximity ligation assay also confirmed PGR/RUNX1 physical protein-protein interaction. Finally, we showed through ATAC-seq and RNA-seq of wildtype *vs* PGR knockout mice that PGR was required to actively enable chromatin accessibility at selective target gene promoters. Overall, we have generated comprehensive maps of epigenomic and transcriptional changes in granulosa cells during ovulation, which provides further understanding of the specialised PGR mechanism in controlling ovulation. Importantly, we showed that direct PGR/RUNX1 interaction is critical for the unique PGR-governed ovulatory network, for which ovulation-targeting novel contraceptives can be designed.

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Leydig Cell Glucocorticoid Receptor is Required to Maintain Steroidogenesis in Adulthood

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Glucocorticoids are used in a wide range of clinical settings, including asthma, eczema, arthritis, Crohn's disease, mental health conditions, and, more recently, COVID-19. An estimated 1 in 2 Australians have received steroid treatment, and this prevalence is likely to increase[1].

Androgens are essential for life-long health and well-being[2]. Disruptions to the production or action of androgens are associated with many chronic pathologies and metabolic disorders. Glucocorticoids, exerting their action via the glucocorticoid receptor (GR), can regulate androgen biosynthesis by blocking key enzymes required for their production in the androgen-producing cells in the testis, Leydig cells[3]. Despite glucocorticoid action on androgen production being well documented, how they regulate testis function is unknown.

To establish the role of GR-signalling in the testis in adulthood, we utilised a novel technique to rapidly generate cell-specific knockouts using an adeno-associated virus to deliver Cre recombinase to Leydig cells specifically. Leydig cell GR knockout mice were generated via injection of Adeno-Associated Virus serous type-9 (AAV-9)[4] carrying either GFP (control) or Cre recombinase into the interstitial compartment of the testis of GR floxed mice. Mice were injected in adulthood and were collected following one round of spermatogenesis.

Our preliminary data show that interstitial delivery of Cre recombinase via AAV-9, is an effective and cell-specific method to ablate GR from Leydig cells. Analysis of blockade of GR signalling in Leydig cells in this model shows markedly suppressed luteinising hormone receptor (*Lhcgr*) and steroidogenic enzymes required for normal androgen production. This important new data suggests that GR-signalling plays a physiological role in normal testis function and potentially fertility. These novel findings provide a timely and previously unavailable opportunity to elucidate the role of GR-signalling in testis function and its ability to influence LC function and androgen production.

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Sexually different mechanisms of meiotic cell cycle in mammalian germ cells

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The mechanisms regulating meiotic initiation in mammals are enigmatic. STRA8, which is expressed in response to retinoic acid, is thought to be a key factor promoting meiotic initiation. However, the specific role of STRA8 in meiotic initiation has remained elusive. Previously, we identified MEIOSIN as a germ cell-specific factor that associates with STRA8 (Ishiguro et al. Dev Cell 2020). MEIOSIN, in collaboration with STRA8, drives meiotic gene activation, and plays an essential role in the switching from mitosis to meiosis.

Here we show that STRA8 binds to RB families, independently of MEIOSIN. To study the physiological relevance of STRA8-RB interaction, we generated mutant mice that express STRA8 lacking RB-binding site (*Stra8*$^{\Delta RB}$), in which STRA8 cannot bind to RB but preserves intact interaction with MEIOSIN. Notably, our genetic study combined with scRNA-seq analysis demonstrated that in *Stra8*$^{\Delta RB}$ female germ cells, progression into G1/S transition was compromised and meiotic entry was concomitantly delayed. This suggests that meiotic initiation is coordinated to coincide with S phase under STRA8-RB interaction in female germ cells. Intriguingly, although *Stra8*$^{\Delta RB}$ oocytes apparently progressed through meiotic prophase, they failed to undergo dictyate arrest, a status of prolonged G2 arrest, resulting in premature loss of oocyte pool. Our study highlights the previously unforeseen implication that oocyte intrinsic program of long-term G2 arrest, is genetically controlled under STRA8-RB interaction.

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TH/INS: A possible marsupial-specific imprinted domain

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Genomic imprinting causes the expression of some genes in a parent-of-origin-specific manner. Comparing the imprinting mechanisms of control between eutherians and marsupials is essential to understand the evolution of imprinting in mammals. More than 80% of eutherian imprinted genes are present as imprinted clusters, allowing genes to share common regulatory elements such as differentially methylated regions (DMRs). In the mouse, an important imprinted domain, the *Igf2/H19* locus, incorporating the insulin-like growth factor 2 (*Igf2*) and the long non-coding RNA, *H19*, is well characterised in the mouse. It is thought that a neighbouring imprinted gene, Insulin 2 (*Ins2*) is also regulated by this common DMR in the *Igf2/H19* flanking region. In marsupials, the *IGF2/H19* imprinted domain is conserved, and Insulin (*INS*) is also imprinted. However, our laboratory previously found a marsupial-specific gene, *TH-INS,* that shares exons of tyrosine hydroxylase (*TH*) and *INS* in the tammar wallaby *Macropus eugenii* [1-2]. The methylation pattern at the *TH-INS* transcription start site (TSS) showed monoallelic expression in tammar pouch young (PY) liver, suggesting it may be a DMR. To confirm this, we first identified a diagnostic single nucleotide polymorphism (SNP) in regions adjacent to the TSS of *TH-INS*. We then tested twelve matched maternal and PY samples and found one homozygous mother with a heterozygous PY. Using these samples we confirmed the presence of DNA methylation at the maternal allele of *TH-INS* TSS in PY liver. We are now investigating the methylation status of the paternal allele. Since the gene shows monoallelic expression in the PY liver we predict that this gene is maternally-imprinted. The proximity of this potential DMR to both *TH-INS* and *INS* suggests that the *TH/INS* region may have evolved as a marsupial-specific imprinted domain.

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Antioxidants in preimplantation mouse embryo culture and vitrification media support a more in vivo-like gene expression profile in fetal liver and placenta post-transfer

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Background:

Assisted reproductive technologies (ARTs), including in vitro culture and vitrification, are known to elicit changes in embryo and fetal gene expression. Previous studies have shown combined antioxidants (acetyl-L-carnitine/N-acetyl-L-cysteine/α-lipoic acid (A3)) in culture and vitrification media reduced the detrimental effects on embryo and fetal development. However, the mechanisms remain unclear therefore the effect of antioxidants on gene expression in mouse fetal tissues was examined.

Design:

Embryo transfers were conducted on in vivo flushed blastocysts, or blastocysts cultured or vitrified with and without A3. Transcriptional profiles of E14 fetal liver and placental tissue in all groups were quantified using RNAseq and functional analyses (KEGG).

Results:

Compared to in vivo derived embryos, in vitro culture altered the expression of 3601 fetal liver and 408 placental genes. Functional analysis showed upregulation/enrichment of oxidative phosphorylation and mitochondrial function and activity. Similarly, vitrification led to 2018 liver and 216 placental differentially expressed genes (DEGs). Upregulated KEGG pathways were enriched for cell and tissue development and cell cycle regulation. Down regulated pathways were associated with metabolism and immune response. Interestingly, A3 in culture media significantly reduced the number of DEGs (1855 liver and 4 placentae) with no KEGG pathways identified. Correspondingly, A3 in vitrification media reduced the number of DEGs to 1017 in liver and 206 in placentae. Functional pathway enrichment was similar to embryos vitrified without A3, although with decreased expression.

Conclusion:

Both in vitro culture under 20% oxygen and vitrification of blastocysts significantly perturbed fetal liver and placental gene expression, with the number of DEGs greater following vitrification. Antioxidants reduced the number of DEGs and biological processes altered, establishing a more in vivo like gene expression profile, particularly in the placenta. Notably, antioxidants significantly reduced gene expression associated with preeclampsia and intrauterine growth restriction which may help maintain the viability of vitrified human embryos.

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New insights into the immunological roles of macrophages adjacent to the rete testis and tunica albuginea

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The rete testis and subcapsular compartment of the testis are sites of disease onset in murine experimental auto-immune orchitis, but macrophages in these compartments are not well-characterised. Macrophages were localised by immunohistochemistry using an anti-F4/80 antibody in testis from adult wild-type mice (n=10-17) and mice with a GFP-expressing transgene at the locus of the macrophage receptor, CX_3CR_1 ($Cx_3cr_1^{gfp/+}$;n=6). Sections were also co-labelled by immunofluorescence for the anti-inflammatory marker, CD206, and the antigen-presenting MHC class II molecule (I-A/1-E) to identify activated macrophages. Sections were scanned (Olympus VS120 slide-scanner), measured (Fiji) and macrophages were enumerated (1-3 sections/animal) using stereological techniques. Compared with parenchyma surrounding the seminiferous tubules, volume density of interstitial macrophages (30 macrophages/μm^3) was 10-fold higher in the rete testis, and the density of peritubular macrophages (3.5 macrophages/μm^3) was 3-fold higher. Macrophage density in subcapsular region was similar to the rest of the parenchyma, and macrophages were also observed within the tunica albuginea itself. In contrast to interstitial macrophages between seminiferous tubules, which lack MHCII expression, most interstitial macrophages in the rete testis and subcapsular region were $CX_3CR_1^+F4/80^+MHCII^{high}$. Peritubular macrophages in the rete testis were $CX_3CR_1^+F4/80^+MHCII^{high}$, but also expressed CD206, unlike peritubular macrophages in the seminiferous tubules. These data indicate that majority of macrophages within the rete testis and subcapsular regions are phenotypically different from macrophages in the rest of the parenchyma in that they have an activated, although anti-inflammatory, phenotype. Accumulation of these macrophages and the anti-inflammatory phenotype of the peritubular macrophages, within the rete testis suggest that they may play a role in recognising sperm antigens and providing protection for mature sperm in the absence of the blood-testis barrier. We predict that loss of the protective function of these macrophages during inflammatory disease may lead to sperm autoimmunity.

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T cells frequency, localisation and interactions during fetal mouse testis development

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It has been well-established that T cells function in inducing immune regulation, immune responses and inflammation in the postnatal testis, but their function and frequency during fetal testis development is undocumented in mouse, rat and human. To study T cells during mammalian testis development, we explored their frequency and localisation in the testis and their contacts with other cells in C57BL6J mice at embryonic day (E) E13.5, E15.5, and at birth (PND0).

PFA-fixed OCT-embedded testes (n=4-7 mice/age) were completely sectioned (6 µM). Four central sections spaced >15 µM were examined using immunofluorescence to detect T cells (CD3), macrophages (F4/80), germ cells (DDX4) and cord basement membrane (laminin). Cell position was designated as in the section perimeter (between capsule and cords) or internal (inside cords or interstitium). Levels of transcripts encoding CD3, F4/80, CD45 (pan-immune cell marker) and DDX4 were measured by RT-qPCR (n=3).

Our study revealed a significant increase in the T cell number from E13.5 to PND0. T cells were absent in the E13.5 testis, rare at E15.5 (0-1/ section), and frequent at PND0 (15±5/ section). RT-qPCR results validated the immunofluorescence data, as *Cd3* was mostly undetectable at E13.5, but increased 2.5-fold from E15.5 to PND0. T cells were detected in the perimeter area, interstitium, and cord perimeter positions. Moreover, the co-localization of T cells (one and/or two) and macrophages appearing as a cluster was a frequent observation at PND0 (2 to 4 clusters/ section).

This study documents for the first time the changing frequency and localisation of T cells during testis development in the fetal and newborn mice and identifies cellular contacts between T cells and macrophages. These results shed light on the presence of T cells and their interactions with macrophages during testis development.

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Spermatozoa interact with ectocervical epithelial cells to modulate cytokine production

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The deposition of seminal fluid into the female reproductive tract at coitus elicits an inflammation-like response characterised by induction of cytokines and chemokines and extensive leukocyte recruitment. In mice, this response is important for preparing the female reproductive tract for successful embryo implantation through promoting immune tolerance towards male allo-antigens. Seminal plasma (SP) has been considered the sole signalling component of semen, however recent mouse studies have shown spermatozoa also contribute to eliciting the female immune response. Whether spermatozoa also contribute to seminal fluid signalling in men remains unclear. In this study, we investigated the capacity of human spermatozoa to interact with immortalised ectocervical epithelial (Ect1) cells to induce cytokine and chemokine synthesis using an *in vitro* seminal fluid signalling model. We observed that washed spermatozoa - as well as whole semen and SP – acted to elicit Ect1 cell production of colony stimulating factor (CSF) 2 (mean of 3-fold increase), CSF3 (5-fold increase), interleukin (IL) 1A (2.3-fold increase), IL6 (1.7-fold increase), CC chemokine ligand (CCL) 2 (1.6-fold increase), CCL3 (1.9-fold increase), and chemokine (C-X-C motif) ligand 8 (CXCL8, 6.6-fold increase). Considerable variation in the profile of cytokines and chemokines induced by sperm from Ect1 cells existed between individual men. Live-cell confocal imaging experiments demonstrated active, reversible binding of subsets of spermatozoa to Ect1 cells. In addition, scanning and transmission electron microscopy showed evidence of close attachment with microvillus structures protruding from the Ect1 cell surface to form intimate associations with the head of the spermatozoa, and in some instances, potential engulfment by Ect1 cells. Currently, the underlying mechanisms and biological significance of

sperm-mediated signalling in cervical epithelial cells is unknown. This preliminary data suggests a previously unknown biological activity of spermatozoa in eliciting the female immune response to seminal fluid.

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Endogenous sphingosine 1-phosphate (S1P) signalling via the $S1P_1R$ is essential for mouse preimplantation embryo development *in vitro*

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The addition of selected growth factors to *in vitro* embryo culture medium improves the viability of cultured preimplantation embryos. One such beneficial growth factor is sphingosine 1-phosphate (S1P). Exogenous S1P improves oocyte maturation (Cheng et al., 2015; Jee et al., 2011) and rate of blastocyst formation (Jee et al., 2011) and decreases rates of apoptosis throughout development (Guzel et al., 2018; Roth and Hansen, 2004). The mechanism by which S1P improves development is poorly understood. The current study aims to determine the expression of the $S1P_1$ receptor ($S1P_1R$) in the mouse preimplantation embryo and its role in endogenous signalling pathways that may promote preimplantation embryo development. Immunofluorescence staining demonstrated that the $S1P_1R$ is expressed in both the plasma membrane and the nucleus in the early stages of preimplantation development and is predominantly within the nucleus at later stages. Within the blastocyst, the $S1P_1R$ was expressed in the trophectoderm cells as well as the hypoblast cells, but not the epiblast cells of the inner cell mass. Activation of the $S1P_1R$ by exogenous S1P in 2-cell embryos resulted in changes in $S1P_1R$ localisation when visualised via immunofluorescence. Most notably, a decrease in nuclear and cytoplasmic $S1P_1R$ staining was observed in embryos treated for at least 5 minutes. Finally, treatment of embryos with NIBR-0213, a potent and selective $S1P_1R$ antagonist, resulted in a dose-dependent decrease in the percentage of embryos that developed to the morula stage and beyond. Together, these findings support the presence and function of the $S1P_1R$ in the mouse preimplantation embryo and suggest that endogenous S1P/$S1P_1R$ signalling is necessary for preimplantation embryo development, though the exact signalling pathways remain to be elucidated.

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Blood plasma-derived small extracellular vesicles as markers of immune dysregulation in cattle: a tool for improving reproductive outcomes

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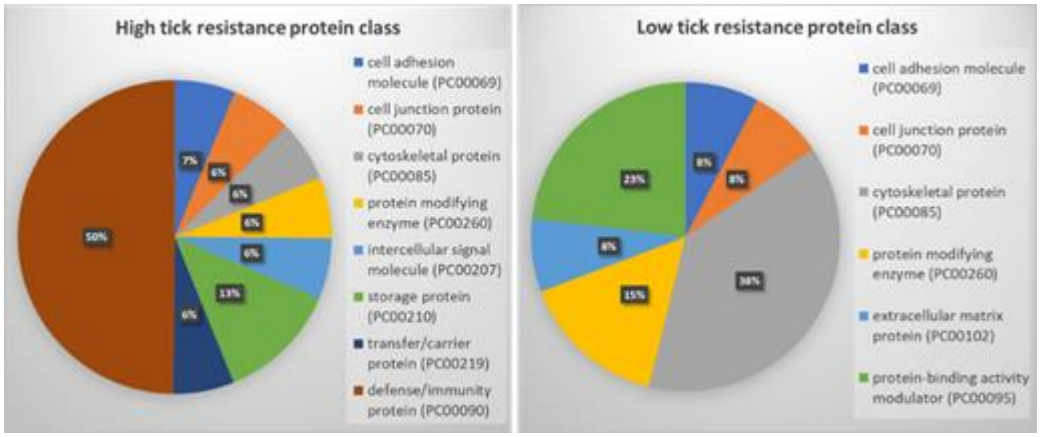
Background: Cattle fertility has been in decline for the past 20 years. Small extracellular vesicles (sEVs) are cell-secreted nanoparticles (~30 – 150 nm diameter) present in biofluids such as blood plasma and have been used as biomarkers of health and disease. As essential players in cell-cell signalling and communication, sEVs provide a systemic snapshot of the overall health state of the animal. Immune dysregulation is associated with poor reproductive outcomes in cattle; therefore, our lab has investigated whether proteomic analysis of circulating (blood plasma-derived) sEVs by mass spectrometry (MS) is a valid tool for profiling cattle with known immune diversity due to tick burden.

Methods: Blood was collected into evacuated EDTA blood tubes from cattle with low (>200 ticks) or high tick resistance (no identifiable ticks) (n = 3/group). sEVs were isolated from blood plasma by sequential centrifugation and size exclusion chromatography using an established method of sEV isolation. sEV and non-sEV containing fractions were pooled separately and subjected to validation experiments prior to being processed for MS analysis operated in data-dependent acquisition mode. Data were processed using Protein Pilot and filtered using 1% false discovery rate (FDR) cut-off at the protein level, and 5% FDR at the peptide level, with minimum 2 peptides per protein. PANTHERGO online software tool was utilised for gene ontology analysis from the final protein list.

Results: A total of 490 unique sEV proteins were identified. Of the 30 proteins unique to high tick-resistant cattle, defense/immunity proteins accounted for 50% of these, however this protein class was not detected in low tick-resistant animals.

Conclusions: Proteome profiling of circulating sEVs is a valid tool for assessing immune status in cattle. Blood plasma-derived sEV profiling may be of use in determining cattle at-risk of immune dysfunction around the time of calving with the aim of improving reproductive outcomes.



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Investigating a potential link between developmental exposures to estrogenic-EDCs and risk of erectile dysfunction

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Erectile dysfunction is an extremely prevalent condition globally and has been estimated to increase dramatically over recent decades. Genetics or increased reporting alone cannot account for this phenomenon, and thus environmental factors have a likely role in the aetiology. As penis development relies on a delicate balance of endocrine signalling, exposure to endocrine-disrupting chemicals (EDCs) may alter patterning of the penis tissue to increase the risk of erectile dysfunction in adult life. Indeed, animal studies have confirmed that exposure to estrogenic-EDCs alters the structure of the corpus cavernosum (CC), a key vascular structure mediating erection via relaxation of the smooth muscle to increase blood flow into the penis.

However, there are currently very few developmental studies examining the links between estrogenic-EDC exposures and erectile dysfunction. Thus, I am exposing developing male mice to the potent estrogen diethylstilbestrol (DES) at a high and low dose which reflects environmentally relevant estrogen exposure. I subsequently rear the males to adulthood and dissect the CC to determine the impact of DES on smooth muscle action using wire myography. I hypothesise that individuals exposed to DES will display altered function of the CC, suggesting a predisposition to erectile dysfunction. This project will lead to a better understanding of the contribution of estrogenic-EDCs to erectile dysfunction, which is critical given the increasing global prevalence of this condition correlates with our exposure to EDCs.

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Chronic butylparaben exposure alters fetal testis gene expression and post-natal growth in male mice

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Endocrine disrupting chemicals (EDCs) are environmental contaminants that contribute to rising infertility rates due to their oestrogenic and anti-androgenic activity. Butylparaben (BuP) is one such EDC widely used in Australia as a preservative in person care products, despite being banned in many other countries. The reproductive impacts of butylparaben exposure remain unclear. The aim of this study was to determine the impacts of environmentally relevant butylparaben concentrations on male fertility using a mouse model. From mating, pregnant C57BL6J mice (n=10 per treatment) were administered vehicle control or butylparaben via sub-cutaneous injection at dosages of 0, 2 or 20 µg/kg/day; mimicking the dermal exposure route and equating to average human exposure levels. Dams were sacrificed at e14.5 (n=6 per treatment) or gave birth, with pups continuing to receive treatments until 3 months of age (n>10 males per treatment). BuP exposure had no effect on maternal weight gain, litter size or sex ratios ($P>0.1$), but exposure to 2 µg/kg/day BuP caused reduced fetal weight ($P<0.01$). Exposure to 2 µg/kg/day BuP also decreased cholesterol side-chain cleavage enzyme (Cyp11a1) and 17β-Hydroxysteroid dehydrogenase (17-β-hsd-1) expression in the fetal testis ($P<0.05$), whilst exposure to 20 µg/kg/day BuP decreased oestrogen receptor 1 expression (Esr1) in the fetal testis ($P<0.05$). In addition, 20 µg/kg/day BuP exposure decreased F1 male bodyweight at 3 months of age ($P<0.05$). Thus, chronic exposure to butylparaben, at levels equivalent to those experienced by human populations, is sufficient to perturb fetal testicular steroidogenic gene expression, as well as reduce male offspring growth rates. These findings support closer evaluation of the safe levels and use of butylparabens, with further studies required to fully elucidate the long-term consequences on male health and fertility.

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Reprograming human fibroblasts into Sertoli cells: functional analysis

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Differences of Sex Development are congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical. Options for functional analysis of variants identified in patients is limited to *in vitro* cell models, biochemical assays and mouse models. Often, the functional consequences of causative variations cannot be elucidated using these methods as there is no cell model that perfectly mimics gonadal cells and there is many key differences between mouse and human gonadal development. We have tried to mitigate this limitation by reprogramming readily available skin tissue derived dermal fibroblasts into Sertoli cells (SC), which could then be used in function assays. We employed a computational predictive algorithm for cell conversions called Mogrify to predict the transcription factors (TFs) required for direct reprogramming of human dermal fibroblasts into SCs. We established trans-differentiation culture conditions where stable transgenic expression of these TFs was achieved in 46, XY adult dermal fibroblasts using lentiviral vectors. The resulting Sertoli like cells (SLCs) were validated for SC phenotype using several approaches. These cells exhibited Sertoli like morphological and biophysical properties that differed as revealed by morphometry and xCELLigence assays. They also showed Sertoli-specific expression of molecular markers such as SOX9, PTGDS, BMP4, or DMRT1 as revealed by IF imaging, RNAseq and qPCR. These cells additionally lacked expression of markers of other gonadal cell types such as Leydig, germ, peritubular myoid or granulosa cells. The trans-differentiation method was also applied to a commercially available 46, XY fibroblast line derived from a patient with DSD. The resulting cells displayed lower levels of SOX9 (a key testis marker) in comparison to normal 46, XY derived SLCs and also showed impaired proliferation in an xCELLigence assay, thus showcasing the robustness of this new trans-differentiation model.

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Blockage of the classical and backdoor androgen production pathways indicates a third pathway in mice

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Androgens are essential for male sexual development, virilisation, spermatogenesis and general health and wellbeing. Androgen deficiency can lead to multiple pathologies including disorders of sexual development, infertility and increased risk of cardiovascular disease and diabetes. Active androgens mainly produced in the testis by Leydig cells include testosterone and the more potent dihydrotestosterone (DHT). Two androgen production pathways have been identified: the classical pathway using testosterone as a precursor to DHT, and the backdoor pathway bypassing the need for testosterone. Both pathways are essential for males, however, it is unknown how they function together, and the importance of each at androgen-responsive endpoints during sexual development and in later life.

Using a knockout (KO) mouse model involving HSD17B3 (classical pathway) and SRD5A1 (backdoor pathway), we are dissecting the roles and interactions between the classical and backdoor androgen pathways (Figure).

Following validation of the model (genotyping, transcript, and hormone levels), we have demonstrated that the double KO of HSD17B3 and SRD5A1 had normal development at birth and adulthood. Testis development at both ages appears normal compared to littermate controls. Epididymal coiling remained present in double KO mice and contained sperm in adulthood. Interestingly, double KO males remain fertile.

Unaltered sexual development and remaining fertile suggest the existence of a compensatory mechanism or a third, unknown, androgen production pathway in mice. We recently identified that HSD17B12 may be involved in this process [1]. Whilst human HSD17B12 is specific to estrogens, mouse HSD17B12 can convert androstenedione to testosterone.

Preliminary observations demonstrate that androgen production is not fully understood. Further investigation is required into deciphering the roles of each pathway in development, reproduction and androgen-related pathologies affecting male health.

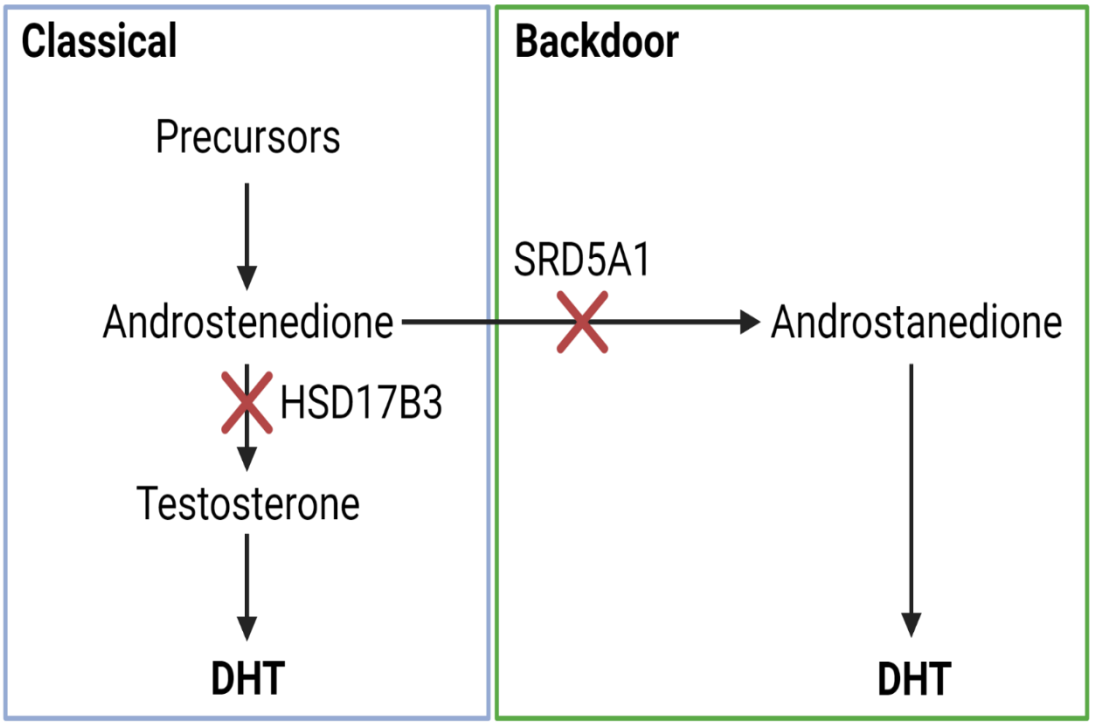

Figure. Schematic representation of the double knockout mouse model. HSD17B3 and SRD5A1 are critical for the classical and backdoor pathways, respectively. X = Knockout of the enzyme.

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Transgenerational effects on male reproduction following exposure to an endocrine disrupting chemical, diethylstilbestrol

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Differences of Sexual Development (DSDs) are amongst the most common birth defects in humans. A rise in the incidence of these disorders has been linked to our increased exposure to endocrine disrupting chemicals (EDCs). In addition, some EDCs are predicted to have far reaching effects beyond the exposed individual, causing disease that persists over multiple generations through alterations to the epigenome. Our study aimed to determine if estrogenic EDCs interfere with the establishment of the germ cell epigenome to cause impacts that persist in subsequent generations. We have examined the effects of diethylstilbestrol (DES), a clinically relevant EDC, across three generations of male mice and analysed the rates of reproductive disorders. A decline in several reproductive parameters including fertility rates, was detected through to the F3 generation in the male descendants of DES exposed mice. A number of DSDs, such as hypospadias and epispadias, were also increased in F1, F2 and F3 generations. This study suggests that exposure of pregnant mothers to DES has direct effects on the developing young, including epigenetic changes to their fetal germ cells, that result in the long-term transgenerational inheritance of male reproductive disorders.

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Utilising human fetal gonad cultures to understand TGFβ superfamily signalling and origins of gonadal pathologies

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Disrupted human fetal testis development may lead to testicular germ cell tumours and other testicular pathologies, particularly during the masculinisation programming window (MPW) which falls between gestational weeks (GW) 8 and 14. During this interval, germ cells undergo epigenetic remodelling and heterogeneously enter quiescence. High levels of transcripts encoding

several TGFβ superfamily members (activin A, activin B, NODAL and LEFTY) are present between GW 10-12. Because fetal exposure to abnormally high activin A levels may occur during pregnancy (e.g. in preeclampsia), we hypothesised that disruptions to TGFβ superfamily signalling *in utero* during the MPW contribute to adult testicular pathologies.

Human fetal gonads were obtained from elective first trimester terminations. Gonads with mesonephros were bisected for 72h hanging-drop cultures, with one half placed in media (MEM-α/10% FBS/1% ITS/1% Pen-Strep) containing 50 ng/mL activin A or 10 μM SB431542 (inhibits activins/Nodal/TGFβ), and one half in media with vehicle. Samples were snap-frozen for qRT-PCR or fixed for histology.

Preliminary results indicate that acute exposure of GW 9 testes to activin A (n=1) or SB431542 (n=2) inversely affected the somatic activin A target transcript *SERPINA5* (activin A, 1.3-fold increase; SB431542, 0.6-fold decrease), and altered the germ cell transcripts *KIT and SOX17*. Limited analysis of GW 8 (n=1) and GW 11 (n=1) testes to date suggests responses are age-dependent. In ovaries, SB431542 increased *KIT* at GW 9-10 (n=4), while activin A reduced *KIT* at GW 9 (n=2), indicating the female gonad is also sensitive to TGFβ signalling disruption.

Ongoing experiments are informed by evaluation of normal human fetal testis transcriptomes from RNA-Seq/scRNA-Seq, and our own datasets from activin A transgenic mouse testes. This will expand knowledge of activin A target genes in human fetal gonads, and ultimately reveal how altered activin A signalling *in utero* may influence adult fertility and gonadal pathologies.

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Exposure to agricultural azoles disrupts retinoid signalling in fetal rodent testes

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Disorders of human male reproductive health include cryptorchidism, hypospadias, infertility/subfertility, testicular germ cell cancer and primary hypogonadism. The 'testis dysgenesis syndrome' hypothesis proposes that ALL of these problems have a shared origin during fetal life: if testis development is perturbed during a critical window of time whilst in the womb, reproductive health and function is affected. These disorders are escalating at such high rates that it is presumed that environmental causes are to blame - the key suspect our increasing exposure to 'endocrine disrupting chemicals', particularly during fetal life.

We have previously shown that the presence of signalling molecule retinoic acid (RA) is detrimental to development of three major cell types of the mouse fetal testis – germ cells, Sertoli cells and fetal Leydig cells. Normally, P450 enzyme CYP26B1 degrades RA, but if CYP26B1 does not function, ectopic RA causes germ cells to aberrantly enter meiosis, the testis is partially feminized, and secondary sexual structures are perturbed.

Combining developmental biology, mouse transgenic expertise, and reproductive toxicology, we have developed a novel ex-vivo testis culture system to read-out RA-Cyp26b1 signalling perturbation. We have used this system to evaluate the molecular effects of a common agricultural azole, Flusilazole, and related compounds. Flusilazole is a fungicide that works by inhibiting the fungal P450 enzyme CYP51, though it is likely that it can also inhibit mammalian CYP enzymes.

Transgenic fetal testes at E12.5 were cultured in hanging drops for 48hrs in the presence/absence of a panel of azole chemicals before harvesting for qRT-PCR analysis, histological examination, staining or imaging. We found that ectopic RA signalling could be detected in response to azole exposure at a range of concentrations, indicating possible consequences for reproductive development and function. Our ongoing work will have future translational importance, in particular for the refinement of current chemical screening methodologies.

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A novel hedgehog signalling network during penis development

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Hypospadias, a failure of appropriate urethral positioning within the penis, is the most common birth defect requiring intervention and can result in lifelong physical and psychological burdens. Surgery is the only therapeutic option for hypospadias, and can fail repeatedly in moderate to severe cases. This is partly because the underlying tissue and molecular signalling networks controlling urethral closure are not fully understood. This study aimed to identify novel networks regulating this process, using a comparative developmental approach.

Marsupials are valuable models to study penis development as gonadal differentiation and subsequent penis development occur after birth. RNAseq, differential expression and gene-ontology analyses were conducted on male and female wallaby penis samples during a critical window of urethral closure, and after treatment with estrogens, androgens and inhibitors of their receptors. The distribution of the hedgehog proteins, Sonic Hedgehog (SHH) and Indian Hedgehog (IHH), as well as the transcription factor SOX9, were assessed in wallaby penis tissue using immunofluorescence. Explants of mouse and wallaby penis were treated with either SHH or IHH, and analysed by qPCR for changes in the expression of genes critical for penis development, to identify any discrete actions of SHH and IHH.

Gene ontology showed enrichment for genes involved in chondrocyte differentiation and bone formation in male samples compared with either female samples, or samples where androgen signalling was interrupted. Key regulators of chondrocyte differentiation, SHH and IHH, are localised to discrete regions of the penis during development, similar to their compartmentalised expression in developing bone. Treatment of explants with SHH or IHH induced distinct expression changes in androgen and

Wnt signalling genes important for urethral closure. Thus, SHH and IHH activate distinct developmental networks during urethral closure, and harnessing these distinct actions may lead to novel treatments that improve outcomes of surgery for hypospadias.

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Gonadal development and germ cell migration in the fat-tailed dunnart (*Sminthopsis crassicaudata*)

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Primordial germ cells (PGCs) are part of a unique and highly conserved cell lineage that gives rise to gametes. In mammals, PGCs arise outside of the embryo and must undergo migration in order to colonise the developing gonad. Once in the gonad, the surrounding somatic environment dictates their differentiation into sperm or eggs. The primary route for PGC migration in eutherian mammals is through the hindgut and dorsal mesentery. However, it has been noted that in marsupials, most PGCs instead migrate through the mesoderm surrounding the hindgut. This finding was interesting and unusual, considering the conserved nature of the germ cell lineage and consistency of their migratory path in eutherian mammals.

We looked at the process of sex determination in the fat-tailed dunnart (*Sminthopsis crassicaudata*), one of the most altricial marsupials at birth. To our surprise, we found that testis differentiation begins at 2 days post-partum (d.p.p.), and clear ovarian differentiation was evident by day 8 d.p.p. This is around the same time relative to birth as seen in less altricial marsupials. Thus, gonad formation in the dunnart occurred at a much earlier developmental stage than seen in other marsupials. We also found that PGCs enter the gonads of both sexes around 2 d.p.p. We noticed that in this species, PGCs appear to be migrating through the hindgut, as they do in eutherian mammals, suggesting that perhaps germ cell migration follows a conserved route after all.

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An inflammatory reaction occurs at attachment during marsupial pregnancy: expression of MUC1 in tammar wallaby uterine epithelial cells (*Macropus eugenii*)

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Embryonic development requires close apposition of the blastocyst to the luminal surface of the uterine epithelium. As the first site of contact between fetal and maternal tissue, the uterine epithelium undergoes specialised changes allowing it to become receptive to attachment. These changes can vary across mammalian species. However, there are general similarities across all live-bearing taxa. In macropodid marsupial pregnancy, this attachment occurs in the last third of pregnancy after the loss of the shell coat which surrounds the developing embryo. This interaction potentially causes a maternal response to the embryo as a foreign body. In this study we have used gene expression data and IHC techniques to characterise the gene expression and localisation of MUC1 in the uterus during pregnancy in the tammar wallaby (*Macropus eugenii)*. We show that, like in human pregnancy, there is an upregulation of MUC1 gene expression at receptivity and attachment which corresponds with localised staining of MUC1 to the uterine epithelium of gravid uteri. The presence of this mucin provides evidence for a maternal inflammatory response in macropodids, like the inflammatory attachment reaction described in the opossum during this crucial stage of pregnancy.

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Characterising the marsupial oviduct: a complete metabolite, protein and hormone profile for the Fat-tailed dunnart during oestrous

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Australia is in an extinction crisis with many of our iconic marsupial species threatened. Assisted reproductive technologies should be a vital part of conservation efforts, yet the fundamental method of *in vitro* fertilisation (IVF) has not been achieved for marsupials. The reason for this is a lack of understanding surrounding the signalling environment within the oviduct at the time sperm meets egg. Marsupial sperm are highly regulated, with research showing sperm will bind to the oocyte only when cultured in media that is conditioned with oviductal epithelial cells. Investigating this further, we used liquid chromatography mass spectrometry (LC-MS) to obtain both the metabolome and proteome for the marsupial oviduct, using the Fat-tailed dunnart as a model. Samples were taken when dunnarts were in oestrous and compared to non-oestrous samples. Interestingly, results suggest fructose is a candidate for the dunnart seminal sugar, with n-acetyl glucosamine (NAG) only lowly detected. Carbohydrate levels reflect what has been observed in previous marsupial embryo work. Serum from each animal was also subjected to LC-MS to obtain a profile for sex steroid hormones, including estradiol and progesterone. We have shown that there is a difference in the oviductal environment during oestrous and plan to use these results to synthesise a media that will better support marsupial sperm capacitation and oocyte binding. The high levels of hypotaurine and L-glutamine during oestrous, as well as the detection of all essential and non-essential amino acids but cysteine, suggest commercial IVF media may be a good starting point, to which additional factors may be added. This is the first study to extensively characterise the marsupial oviductal environment and will be a fundamental resource for the field of marsupial IVF moving forward.

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Characterisation of the candidate monotreme sex determining gene Y-localised Anti-Müllerian Hormone (*Amhy*)

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The egg-laying mammals (monotremes) form the most basal, extant mammalian lineage and possess both mammalian and reptilian features. Most intriguingly, the monotremes (platypus and echidna) have a multiple XY sex chromosome system with 5X and 5Y chromosomes in the male platypus and 5X and 4Y in the echidna male. These multiple sex chromosomes are unique to this mammalian group, having evolved after divergence of the monotremes from therian mammals. Although monotremes possess genes common to the vertebrate sexual differentiation pathways, they lack the master sex determinant (MSD) gene, *Sry* required for testis development in therian mammals. The monotreme MSD is unknown. In a number of teleost fish, a Y chromosome specific Anti-Müllerian hormone (*Amh*) gene has been recruited independently to the role of MSD. Monotremes are the only mammals known to carry the *Amh* gene on sex chromosomes (bearing an X and Y copy). As such, the Y localised *Amh* gene (*Amhy*) is the primary candidate MSD in monotremes. Here we characterise the monotreme *Amh* genes showing that the Amhx and Amhy proteins contain the conserved features and predicted structures of other Amh proteins suggesting that they also function through Amh receptor II signalling. We demonstrate that the *Amhy* gene and protein of monotremes has significantly diverged from its X-localised gametologue. Differences between the *Amhy* and *Amhx* promoters and introns may underlie differential expression of the genes and support the posited role of *Amhy* as the monotreme MSD.

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Changes in the pouch microbiome during lactation of the short-beaked echidna (*Tachyglossus aculeatus*)

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The short-beaked echidna is the most widespread native Australian mammal and is of ecological and evolutionary importance. Echidnas are also commonly kept in zoological gardens but are often difficult to breed in captivity. The reproductive biology of short-beaked echidnas is unique as one of only three egg-laying mammals (alongside the platypus and long-beaked echidna), where the young hatch from their eggs in the pseudo-pouch in which they reside and are nursed by milk secreted from the milk patch. Previous studies on marsupial pouches have revealed changes in the microbial environment during lactation. To investigate whether the microbiome changes during lactation in echidnas, swab samples were obtained from wild and captive echidna pouches from three different time periods within the reproductive process: outside of breeding season, during courtship and breeding, and during lactation. We found that the pouch microbiome shows dramatic changes during lactation compared to inside and outside of breeding season, with a reduction in bacterial species that may be pathogenic. This suggests that, like marsupials, the pouch environment changes during lactation to accommodate young with an underdeveloped immune system. This study pioneers pouch microbiome research in monotremes, provides new biological information on echidna reproduction, and may inform captive breeding in the future.

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Folliculogenesis and oocyte survival in mammalian ovaries is dependent on ATP6AP2

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In the mammalian ovary, the proper assembly, activation and quiescence of follicles is crucial for oocyte survival and reproduction, and depends on the concerted activities of multiple signalling pathways, including WNT. The Pro(renin) Receptor (PRR), encoded by the X-linked *Atp6ap2* gene, is best known for its role in the renin-angiotensin system. However recently, its importance as a mediator of canonical WNT signalling has emerged based on its function as a bridge between the WNT receptor LRP6 and the vacuolar H+-ATPase (V-ATPase). These findings, together with our observation that *Atp6ap2* is highly expressed in the developing gonads, led to our hypothesis that PRR is important for ovarian development. To investigate the function of PRR in the developing ovary, we generated ovarian somatic cell-specific *Atp6ap2* conditional knockout mice. Here, we present the ovarian phenotype of these mice and show that PRR is essential for folliculogenesis and oocyte survival, and is linked to diverse cellular mechanisms in the ovary including cell differentiation, cell polarity, gap/adherens junction formation and autophagy. Our findings therefore suggest PRR plays a pleiotropic role in the developing mammalian ovary.

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TOP3A is required for the maintenance of the ovarian follicular reserve and oocyte quality

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Several lines of evidence from budding yeast, plants and flies suggest that TOP3A is critical for quality control in oocytes, with roles in the repair of meiotic DNA double strand breaks and DNA damage arising from exogenous stressors, as well as in the maintenance of mitochondrial DNA (mtDNA). Whilst these studies are compelling, the role of TOP3A in the oocytes of higher vertebrates has never been established. Hence, this study aimed to use novel mouse models to define the functional impact of the conditional loss of TOP3A in oocytes (cKO: TOP3A$^{fl/fl}$;GDF9$^{cre/+}$).

In situ hybridisation using ovarian tissue sections from WT (TOP3A$^{fl/fl}$;GDF9$^{+/+}$) animals revealed that *Top3a* mRNA is expressed by oocytes within primordial, primary, secondary and antral follicles. Furthermore, analyses of WT oocytes injected with GFP-tagged TOP3A protein demonstrated that TOP3A co-localises with mitochondria during oocyte maturation.

Ovarian follicles were subsequently quantified in prepubertal (postnatal day 20) and adult (postnatal day 60) WT and TOP3A-cKO mice (n=6 mice/genotype/age). Follicle numbers were similar in prepubertal WT and TOP3A-cKO mice, but loss of TOP3A resulted in the dramatic depletion of follicles in adults, indicative of premature ovarian aging (follicle number: WT 2367±463 vs TOP3A-cKO 0, p=0.007).

To gain further insight into the requirement for TOP3A for oocyte quality, *in vitro* fertilisation (IVF) and embryo culture were performed using mature oocytes obtained from WT and TOP3A-cKO mice at PN20 (n=6 mice/genotype). Strikingly, almost all oocytes from TOP3A-cKO mice failed to progress beyond fertilisation, whilst fertilisation and embryonic development progressed normally when oocytes from WT mice were used.

Collectively, these data demonstrate that TOP3A is essential for the maintenance of the ovarian follicular reserve and oocyte quality. Although the precise roles played by TOP3A are not yet known, future studies will investigate the hypothesis that TOP3A is a central mediator of mtDNA homeostasis in oocytes.

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Optical assessment of the effect of aneuploidy on oocyte and embryo metabolism.

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Publish consent withheld

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Identifying the cell cycle-related factors in the pregranulosa cells driving primordial follicle activation

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Female fertility is controlled by the number of oocyte-containing follicles within the ovary. These follicles (termed primordial follicles) are established early in development and must sustain reproductive function across a lifespan. Primordial follicle activation is the first step in the selective development of mature follicles for ovulation. The mechanisms that control the activation of primordial follicles are still largely unknown. We used single-cell RNA sequencing to examine the transcriptional profile of mouse embryonic and neonatal ovaries at three timepoints: embryonic day (E) 18.5, postnatal day (PND) 4 and PND7, which correspond with primordial follicle formation and activation. In total, 24,810 cells were sequenced, and we identified 5 distinct clusters of granulosa cells. We distinguished the transcriptomic signature of pregranulosa cells during follicle activation in granulosa cell cluster 1 (Gc_1), by comparing with the activated granulosa cells present in granulosa cell cluster 4 (Gc_4). 389 genes were upregulated in the Gc_1 cluster, compared with 278 genes in Gc_4, and these differentially expressed genes were annotated to divergent cell cycle processes. To validate the role of cell cycle in regulating follicle activation, we performed transcriptomic analysis of a mouse line deficient in *Cdkn1b*/p27^{kip1} (a cell cycle inhibitor), which shows a follicle activation phenotype (Rajareddy *et al.*2007). The gene expression signature identified in pregranulosa cells in our single cell RNA sequencing analysis was observed precociously in the infertile *Cdkn1b*$^{-/-}$ mouse line. Combined, this data suggests that a loss of cell-cycle regulation in pregranulosa cells results in precocious primordial follicle activation.

This dataset provides the groundwork for characterising a gene regulatory network that regulates primordial follicle activation. An understanding of the factors that act co-operatively to stimulate/inhibit follicle activation will reveal key insights into how the

ovarian reserve is established and how this may be dysregulated in female infertility disorders and premature reproductive decline.

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A comparison of the fatty acid composition of equine follicular fluid from different sized preovulatory follicles.

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An IVF protocol has not been established in the horse, potentially due to inefficient oocyte maturation and sperm capacitation methods. During in-vivo oocyte maturation, follicular fluid (FF) envelops the oocyte serving as a molecular reservoir of metabolic activity. Fatty acids (FAs) found in FF are incorporated by the oocyte influencing maturation and quality. During ovulation some FF bathes the oviductal epithelium, and if present spermatozoa. To better understand the oocyte maturation milieu and sperm capacitation, the FA composition of FF from preovulatory follicles of increasing diameters were examined.

FF was aspirated from slaughterhouse-derived ovaries of healthy, cycling mares, and follicle diameter was measured using vernier-callipers. FF was aspirated using a 22-gauge needle, centrifuged and stored at −80°C. FA profiles were analysed via direct transesterification, followed by gas chromatography (GC), on a Shimadzu GC2010, using a fused-silica capillary column.

Individual FA concentrations were reported as percentage, with 14 FAs identified. Palmitic (mean±SD: 30.58±2.44), stearic (26.19±2.15), oleic (15.19±2.54) and linoleic acids (16.02±1.96) had consistently high concentration and small variation across all follicles. Follicles (n=11) ranged from 9.1mm-50mm and were divided into groups according to diameter: small (n=5, <18mm) and large (n=6, >18mm). Only arachidonic acid (AA) levels were significantly higher in large follicles (mean±SEM: 3.69±0.39) compared to small (1.82±0.61), t-test=2.70, p=0.02.

AA-derived metabolites, especially prostaglandins, influence granulosa-cells steroidogenesis and follicle rupture mechanisms, acting as cell-signalling intermediates in cAMP activation and PI3K/AKT. As such, they may contribute to oocyte maturation and the granulosa gap-junctions breakdown preceding ovulation. Saturated FAs have detrimental effects on oocyte maturation, two of which (palmitic/stearic) where in high concentrations across all follicles. Except for the polyunsaturated AA, these results indicate equine preovulatory follicles contain high steady-state FA concentrations, regardless of size. Research is required to further examine the role of equine FAs during oocyte maturation and sperm capacitation.

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Investigating the molecular control of oxygen supply during oocyte maturation

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Oxygen is vital for oocyte maturation, however oxygen regulation within ovarian follicles is not fully understood. The erythrocyte protein haemoglobin is abundant within the *in vivo* matured oocyte, but significantly reduced following *in vitro* maturation (IVM)[1], indicating potential function as an oxygen regulator. The molecule 2,3-bisphosphoglycerate (2,3-BPG) facilitates the release of oxygen from haemoglobin in erythrocytes. This interplay between 2,3-BPG and haemoglobin has been demonstrated in non-erythroid tissues[2,3], suggesting the same might occur in the oocyte[4]. Towards understanding the role of 2,3-BPG in the oocyte, we characterised the gene expression and protein abundance of bisphosphoglycerate mutase (Bpgm), which synthesizes 2,3-BPG, and whether this is altered under low oxygen or haemoglobin addition during IVM.

We quantified haemoglobin and Bpgm expression within *in vivo* matured human cumulus cells and mouse cumulus-oocyte complexes (COC) to determine physiological levels of Bpgm. Next, we evaluated whether Bpgm gene expression and protein abundance was dysregulated during standard IVM of mouse COCs at 20% oxygen. Finally, we attempted to normalise Bpgm expression in the presence of low oxygen (2% or 5% oxygen) and/or exogenous haemoglobin, to mimic the *in vivo* environment.

Our investigation revealed that Bpgm expression was significantly lower than haemoglobin in both *in vivo* matured human cumulus cells (49-fold, $P<0.0001$) and mouse COCs (15-fold, $P<0.005$). Intriguingly, following IVM at 20% oxygen, Bpgm gene expression and protein abundance was significantly elevated compared to *in vivo* (11-fold, $P<0.005$). Moreover, in the presence

of 2% oxygen, Bpgm expression was further elevated compared to 20% oxygen (2-fold, $P<0.05$). This was reduced by haemoglobin addition albeit not to *in vivo* levels (3-fold, $P<0.05$).

Our study demonstrates that IVM results in dysregulation of Bpgm that cannot be normalised by low oxygen or haemoglobin addition. These findings offer further insight into the potential role of haemoglobin as an oxygen regulator during IVM.

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NMN supplementation rescues fertility and bone strength in chemotherapy-treated mice

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Cancer survivors can face infertility from cytotoxic chemotherapy drugs, which exhaust the follicular reserve, leading to endocrine disruption. This can cause infertility with premature menopause and declining bone health. We assessed the effects of the NAD^+ precursor nicotinamide mononucleotide (NMN) on ovarian function, fertility, hormone levels, and late-life bone health. Seven-day old female mice were treated +/-cisplatin (2 mg/kg) and two weeks later received NMN (2 g/L) in drinking water, persisting throughout life. A breeding trial was conducted at 6 weeks, and animals were sacrificed at 24 months to assess late-life effects on bone structure. This experiment was then replicated with collection at 3, 6, and 10 weeks of age to assess early life impacts on bone. Cisplatin caused a dramatic decline in all fertility endpoints. NMN supplementation rescued the cumulative number of pups born per female in cisplatin treated animals by 5-fold (p=0.015). Given the link between estrogen and osteoporosis and the improvement in breeding, we tested whether these interventions impacted late-life bone health at the age of 24 months. Bones from these animals were subject to mechanical and structural analysis to assess osteoporosis onset differences. In cisplatin treated animals, NMN rescued cortical bone thickness, bone volume, and bone density to control levels, and increased mechanical strength. Cisplatin caused striking differences in cortical porosity and matrix organization with consistent rescue by NMN. Surprisingly, in the young animal cohort, in 3-week old mice, cisplatin improved bone trabecular volume, thickness and BV/TV, and cortical volume, density, and wall thickness. Estrogen levels were undetectable at 3 weeks, but progesterone was increased with NMN+/-chemo, with preliminary histology suggesting a higher number of corpora lutea. Together, long-term NMN treatment delivered following chemotherapy protected against ovarian failure and infertility, and notably improved late-life bone health, possibly due to the prevention of premature ovarian failure.

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Chronic multi-generational atrazine exposure effects female mouse health and fertility

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Understanding the effects of pervasive environmental toxicants on the ovary, oocytes, and female fertility is vital, as perturbations can result in reproductive disorders and systemic diseases in current and future generations. The herbicide atrazine (ATZ) is a common ground and surface water contaminant in Australia and globally. Exposure to high atrazine concentrations can significantly effect metabolic health and reproductive processes. However, comprehensive analyses of the impact of environmentally relevant exposures on ovarian function and female fertility, as well as multigenerational effects are lacking. Thus, we have continuously exposed female mice to an environmentally relevant atrazine concentration (0.02 ng/ml; a conservative contamination level in Australian waterways), via their drinking water for three generations. Data from the first generation indicates there was no significant difference in females body composition, however there was a significant reduction in liver mass (ATZ 0.038±0.0091, control 0.045±0.0036, n=13, P=0.019) and bone mineral density (ATZ 0.0536±0.002186, control mean 0.05568 ±0.001392, n=9-13, P=0.013) in females exposed to atrazine compared to controls. In the ovary, the primordial follicle reserve tended to decrease (ATZ 85±30.6, control 118.6±19.6, n=5, P=0.073) and the number of atretic antral follicles increased (ATZ 7.6±4.037, control 3.2±1.304, n=5, P=0.049) in ATZ-exposed females. Interestingly, blastocysts from ATZ-exposed females, produced 1.88 ± 1.2-fold higher levels of ATP relative to controls (n=25-32, P<0.001), a possible indicator of stress and longterm effects. These data suggest that while very low doses of atrazine in our waterways may not manifest in obvious metabolic health defects (e.g. obesity), there may be subtle impacts on liver and bone health as well as oocyte quality. To determine if these effects are compounded by chronic atrazine exposure, female mice from second and third generations will also be analysed. This

study represents the first in-depth analysis of the impacts of chronic multi-generational atrazine exposure on female reproductive health.

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Dysfunction of macroautophagy and mitophagy in the aged oocyte

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The precipitous age-related decline in human female fertility is accompanied by an increase in poor-quality oocytes within the ovary. The autophagy pathways including macroautophagy, mitophagy, and chaperone-mediated autophagy are essential mechanisms of protein degradation responsible for maintaining cell health and promoting healthy ageing. However, there is a limited understanding of these pathways in the context of oocyte health, ageing, and ultimately human infertility. To investigate this, oocytes were collected at young (4 week-old) and aged (12 month-old) time points from C57BL/6 x CBA mice before being assessed for the presence and functional competence of autophagy pathways. Using comprehensive image analysis of macroautophagy pathway markers, we have previously demonstrated the impairment of the macroautophagy pathway in aged oocytes, harbouring a reduction of autophagosome and lysosome number. This was accompanied by an accumulation of large amphisomes (greater than 10 μm^2 in area) in aged oocytes that was mimicked in young oocytes treated with lysosomal inhibitor chloroquine1. In this study we assess another autophagy pathway, mitophagy, by initially assessing the markers microtubule-associated protein 1 light chain 3B (LC3B; a constituent of the autophagosome membrane), and translocase of outer mitochondrial membrane 20 (TOMM20). Increased co-localisation of LC3B and TOMM20 markers in aged oocytes was evident indicating a potential increase in mitophagy with ageing ($P < 0.05$). These findings may provide a link between autophagy pathway dynamics and the known phenomenon of age-related mitochondrial dysfunction within oocytes. Overall, these data highlight dysfunctional autophagy pathways and lysosomal mechanisms as important contributors to the deterioration of oocyte quality in aged mice. Further characterisation of autophagy pathways and the mechanistic basis by which they regulate oocyte quality may ultimately inform the development of therapeutic strategies to maintain pre-ovulatory oocyte health, particularly in older women.

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Insights into the NAD^+ biosynthesis pathways involved during the in vitro maturation of porcine oocytes

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Nicotinamide adenine dinucleotide (NAD^+) is an essential cofactor in many cellular processes and its role in the production of energy is well characterised. Nicotinamide mononucleotide (NMN) is a precursor for NAD^+ biosynthesis and NAD^+ elevating treatments have been found to improve oocyte quality in cattle, mice and pigs [1-3], suggesting that NAD^+ is vital during oocyte maturation. The aim of this study was to examine the effects of inhibiting nicotinamide mononucleotide adenylyltransferase (NMNAT), a key enzyme in both the Preiss-Handler and salvage pathways of NAD^+ biosynthesis, on the maturation of porcine oocytes in vitro. Porcine oocytes from small antral follicles were matured for 44 h in defined maturation medium either without (control) or with gallotannin (55 µM; NMNAT inhibitor), NMN (100 µM) or gallotannin and NMN combined. At 44 h, maturation rates were determined, and mature oocytes were fixed and stained to assess spindle assembly and chromosome segregation. Inhibition of NMNAT reduced the proportion of oocytes that reached MII (25.59 ± 1.13 vs. 84.67 ± 0.03 and 93.89 ± 3.09%; $p < 0.0001$), reduced spindle length (0.38 ± 0.38 vs. 6.56 ± 0.29 and 6.72 ± 0.45 µm; $p < 0.001$) and decreased spindle width (0.90 ± 0.90 vs. 9.14 ± 0.38 and 7.86 ± 0.50 µm; $p < 0.0001$) compared with control and NMN supplemented oocytes. Co-supplementation of gallotannin with NMN rescued spindle width to a diameter similar to that of control oocytes (7.36 ± 3.16 µm; $p > 0.05$) but had no effect on maturation rates or spindle length. The results show that inhibition of NMNAT by gallotannin dramatically impaired oocyte meiotic progression and spindle assembly. The finding that co-supplementation with NMN partially restored spindle assembly indicates that NAD^+ production via the salvage pathway is involved in this crucial process.

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GM-CSF during in vitro oocyte maturation increases mitochondrial activity in cumulus oocyte complexes

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We have previously shown that the addition of Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) during *in vitro* oocyte maturation (IVM) can improve embryo development. The present study was undertaken to understand if the addition of GM-CSF during IVM improves mitochondria membrane potential of oocytes. C57Bl6 x CBA F1 female mice age 21-23 days were injected with 5IU of eCG and cumulus-oocyte complexes (COCs) aspirated from large antral follicles 46-48 h post-injection. Ten COCs were cultured per 50µL drop in bicarbonate-buffered α-MEM containing 3 mg/ml BSA,1 mg/ml Fetuin and 5 mIU/mL FSH plus 0 or 10ng/ml of GM-CSF. 16 hours later matured COCs and denuded oocytes were incubated for 15 minutes in HEPES buffered alpha-MEM media with JC-1 dye (5, 5_,6,6_-tetrachloro- 1,1_,3,3_ tetraethylbenzimidazolyl-carbocynanine iodide; Molecular Probes, OR, U.S.A.). After 15 minutes of incubation stained oocytes/COCs were washed to remove excess stain and then mounted on a glass slide for imaging. The mean red and green intensities were analysed in four different regions within three areas of oocyte: outer area, intermediate area, and peri-nuclear area. Fluorescence was visualised in Cell Voyager CV1000 Confocal Scanner (Yokogawa, Japan) and images were analysed using Fiji Image J software. The experiments were replicated three times with 45-60 COCs/oocytes per treatment group. Instrument settings were kept constant for each replicate. Data were analysed using a univariate general linear model in SPSS. The addition of GM-CSF during IVM had no effect on mitochondrial membrane potential in denuded oocytes, however, there was a significant increase in mitochondrial membrane potential in mature COCs (2.42 vs 1.92; P <0.01). In conclusion, we have shown that the addition of GM-CSF during IVM increases COC mitochondrial activity which may contribute to increase embryo development seen previously.

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The effect of colony stimulating factor 1 on in vitro oocyte maturation

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Colony Stimulating Factors (CSF-1, -2, and -3) are found in the reproductive tract and have been shown to have beneficial effects when added to embryo culture media in a variety of species including humans. CSFs are also found in the ovarian follicle suggesting they may have a role in oocyte maturation. We have recently shown that CSF2 can improve in vitro oocyte maturation, however the role of CSF-1 and CSF-3 has not been determined. The present study was undertaken to determine if CSF-1 - also known as Macrophage-Colony Stimulating Factor (M-CSF) can improve in vitro oocyte maturation (IVM). To determine this a dose response experiment using doses shown previously to improve embryo development when added to embryo culture media, was undertaken and preimplantation embryo development examined. Female mice were injected with eCG and cumulus oocyte complexes (COCs) aspirated from large antral follicles 46-48 h post injection. COCs were matured with either 0, 2 or 10ng/ml of CSF-1. Following fertilization, presumptive zygotes were cultured and embryo development examined, with day 5 blastocyst cell numbers determined by differential staining. No difference was seen between the control, 2 and 10 ng /ml groups in cleavage rate (mean 94% vs 94% vs 90% respectively) or day 4 morula rates (22 vs 18.5 vs 19.7), and day 5 blastocyst rate (68.6% vs 74.6% vs 74%). There was also no difference in day 5 blastocyst inner cell mass cell numbers (16.6 vs 17.7 vs 17.7), trophectoderm (41.4 vs 41.4 vs 43.3) or total cell numbers between the three groups. In conclusion our results suggest that adding CSF-1 during IVM does not improve oocyte developmental competence.

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Follicular Fluid Aspiration and Oocyte retrieval techniques, Several Flushing, difficulty Situations Damage risk to the cumulus complex and complications

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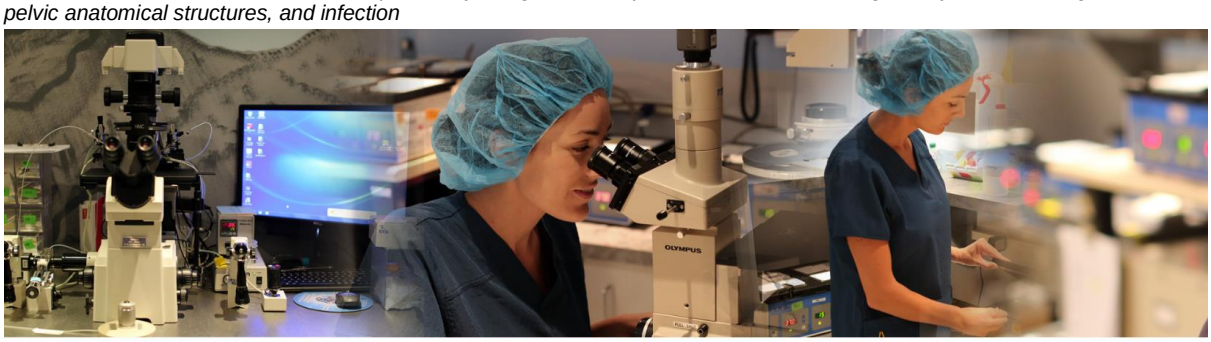
In Assisted Reproductive Technology (ART), oocyte retrieval is a vital step. It was initially performed through laparoscopy, which was complex and difficult and of low efficiency. Ultrasound-guided transvaginal oocyte retrieval was safer and more effective; it is presently the standard operation for in vitro fertilization (IVF) treatment. Theoretically, oocyte retention is achievable after the initial aspirate due to abnormal development of the follicle or oocyte and human technical factors, and such retention could be overcome by recurrent follicular flushing. Follicular flushing is considered to maximize the number of oocytes retrieved and thereafter to improve the rate of IVF pregnancy. There are a number of factors that can affect egg collection and/or egg damage. These involve variables like pump vacuum flow, velocity; needle bore size and length, follicle pressure and size, and collection

techniques. Cook Medical Technology, Brisbane, has developed appropriate equipment to study the factors influencing the success of egg collection and the cause of egg trauma. Experimental & physical aspects of oocyte retrieval Apart from a comparison between manual and mechanical suction on the impact of zonal damage , surprisingly little has been reported on oocyte collection theory until the studies carried out by Cook Medical Technology, Brisbane . A variety of variables may impact oocyte collection and/or ovary damage. These include variables like vacuum flow of the pump, velocity, size and length of the needle lumen, follicular pressure and size and collection techniques. Cook Medical Technology, Brisbane, established appropriate equipment for the study of factors affecting the performance of oocyte collection and the cause of oocyte trauma . Complications The ultrasound-guided transvaginal technique is a very efficient and simple procedure. However, this should not distract from the fact that a number of potentially dangerous complications exist, consisting mainly of hemorrhage, trauma to pelvic anatomical structures, and infection



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Glucocorticoids prevent normal flow deceleration in feto-placental vasculature and impair placental oxygen diffusion during late pregnancy in the rat

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Optimal development of placental vasculature is fundamental to placental function and fetal growth. The structure of the fetal side of the placental circulation determines both biomechanical forces (shear stress and pressure) on the endothelium, and blood flow deceleration towards the capillary level to facilitate capillary oxygenation. To better understand these relationships, we used a well-established rat model of fetal growth restriction. Pregnant Wistar rats were administered dexamethasone acetate (Dex) from embryonic day (E)13.5 until E21.5. Three-dimensional feto-placental vascular casts were obtained from control (n=8) and Dex (n=8) pregnancies. These were analysed for structural changes and the 3D geometries were used for computational fluid dynamics simulations with high-frequency Doppler ultrasound measurements implemented as input to the model. Biomechanical forces, downstream velocities and oxygenation were extracted as mean time-averaged values.

Dex reduced fetal (-21%; p<.0001) and placental (-36%; p<.0001) weights relative to controls. Placental tissue volume (-39% p<.01) and vessel volume (-53% p<.001), number (-62% p<.05) and length (-48%; p<.05) were also reduced. There were minimal differences in umbilical artery Doppler velocity, yet simulations revealed terminal velocities near the capillary bed were 57% (p<.05) greater in Dex placentas relative to controls and consequentially, overall Dex placental oxygenation was 69% (p<.05) lower. Shear stress and pressure were 46% (p<0.05) and 86% (p<0.05) higher in Dex placentas, with differences pronounced in smaller vessels.

The reduced vascular complexity of Dex placentas prevented normal blood flow deceleration, hence reducing oxygen diffusion. The observed increase in shear stress may indicate flow-mediated vasodilation occurs as a response to impaired vascular structure, but at the expense of flow deceleration. Moreover, the homeostatic response to increased pressure could cause increased arterial stiffness, previously reported in growth-restricted fetuses. Ongoing work involving fetal-placental gene expression and other gestational timepoints will provide a deeper understanding of placental function and fetal growth restriction.

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Post-term pregnancy is associated with increases in senescence and mitochondrial adaption through elevated MFN1 and MFN2 expression in placental tissue.

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BACKGROUND: The placenta is a unique organ that despite constant influences by external factors, has a defined life span of approximately 40 weeks in humans. This is the result of aging, which mitochondria play an important role, however its role of aging in a healthy pregnancy remains elusive. This study aimed to identify aging markers and the role of mitochondria within healthy placentae collected from term and post-term pregnancies.

METHODS: Term (39-40 weeks' gestation, n=6-7) and post-date (41-42 weeks' gestation, n=6-7) human placental samples were taken at delivery from the Gold Coast University Hospital, Queensland. Following RNA extraction, qRT-PCR was utilised to measure genes to assess the profile of cellular stress and dysfunction associated with gestational aging. Subsequent protein extraction and western blotting was performed on proteins of interest. Gene and protein markers of aging and mitochondrial processes were measured via qPCR and western blotting.

RESULTS: This study found increases of senescence marker p53 and anti-apoptotic marker BCL-2 were observed in post-term placenta. Gene expression of *SIRT1*, *SIRT3*, *FOXO1* and *TFAM* were decreased in post-term tissue, however, mitochondrial complex I, III, IV and V were increased, possibly due to an observed increase in mitofusins MFN1 and MFN2 protein expression. The increase in citrate synthase/mitochondrial content in post-term placenta is suggestive of an increase in mitochondrial adaptations in healthy post-term placenta.

DISCUSSION: These observed changes in mitochondrial adaptation and aging markers may be the reason why some placental tissue can progress to post-term, whilst others can turn pathological.

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Nutrient-sensing components of the mouse intestine during pregnancy

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Background: Increased maternal food intake during pregnancy is essential for normal fetal growth, while under- or over-nutrition increases pregnancy complications and adult disease risks in progeny. Meal termination occurs, at least in part, through nutrient induced release of satiety hormones from intestinal enteroendocrine cells (EECs). This occurs upon activation of specific EEC nutrient-sensors by luminal carbohydrates, fatty acids and proteins. It is unknown whether intestinal nutrient-sensing adapts to facilitate increased food intake during pregnancy.

Aims: To measure food intake and intestinal expression of nutrient-sensors and satiety hormones across pregnancy in mice.

Methods: Female C57BL/6J mice (10-12wk) were mated and randomised to early- (6.5d), mid- (12.5d) or late-stage pregnancy (17.5d) groups, or age matched, non-pregnant controls (N=10-12/stage). Mice were housed in Promethion cages for the duration of pregnancy to monitor body weight and food intake behaviour. Quantitative RT-PCR was used to determine the relative expression of intestinal fatty acid, protein and carbohydrate sensors and hormones in the duodenum, jejunum and ileum across pregnancy groups (N=6-8/stage).

Results: Pregnant mice were heavier from day 7 and ate more by mid-pregnancy, predominantly due to an increase in meal size in the light phase ($P<0.05$). The duodenal expression of transcripts for free fatty acid receptor 4 (FFAR4) and the protein receptor, GPR93, was lower ($P<0.05$) and for FFAR2 was higher ($P<0.05$) as pregnancy progressed. Transcript levels for cholecystokinin and pro-glucagon were unchanged in the duodenum across pregnancy.

Conclusion: Maternal food intake increases during pregnancy in support of fetal development. Region-specific reductions in intestinal FFAR4 and GPR93, if present as proteins, may reduce fatty acid and protein sensing and the subsequent deployment of satiety hormones, to promote food intake during pregnancy. Understanding the plasticity of nutrient-sensing in pregnancy has the potential to yield new strategies that optimise maternal nutrition and safeguard the lifespan health of progeny.

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New human models of preeclampsia offering physiologically relevant assays for the development of therapeutics to ameliorate vascular dysfunction that occurs in preeclampsia

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OBJECTIVE: The onset of maternal hypertension is a hallmark feature of preeclampsia, resulting from widespread endothelial dysfunction and systemic vasoconstriction. Central to this study, we set out to create a new model that mimics the vascular dysfunction in preeclampsia to evaluate new therapeutic strategies.

METHODS: Human omental arteries were collected from normotensive pregnant women at term (n=9). Serum was collected from women with pregnancies complicated by preterm preeclampsia (delivery <34 weeks gestation, n=8), term preeclampsia (delivery >37 weeks gestation, n=5) and healthy gestation matched controls (n=15). We performed *ex vivo* whole vessel wire myography (DMT 620M) to investigate the vascular effects of treatment with serum from these pregnancies. Omental vessels were treated with increasing doses of serum (2-20%) to assess vasoconstriction. Vessels pre-constricted with preterm serum were then treated with esomeprazole (0.1-100uM), a candidate therapeutic for preeclampsia.

RESULTS: All vessels constricted in response to pregnant serum. There was no significant difference in constriction between human omental arteries in response to either preterm or term preeclamptic serum, compared to gestation matched controls. Esomeprazole treatment did not alter omental artery vasorelaxation in response to normotensive serum. However, esomeprazole treatment significantly enhanced vasorelaxation of arteries pre-constricted with preterm preeclamptic serum compared to vehicle control.

CONCLUSION: Here we developed sophisticated human physiological models of preeclamptic vascular constriction to better test therapeutic strategies. These data demonstrate the exciting potential of our new models; esomeprazole, a novel therapeutic candidate, directly enhances vascular relaxation following constriction driven by serum derived preeclamptic mediators.

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Establishing of a novel placental-on-a-chip model

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Background: Preeclampsia is a cardiovascular disorder diagnosed post 20 weeks' of gestation. It is the leading cause of morbidity and mortality in pregnancy. Inappropriate placental development and growth due to aberrant angiogenesis and inflammation are the root causes of preeclampsia. However, due to ethical limitations and inadequacy of preeclampsia models, the molecular mechanisms regulating these processes are poorly understood. In this study, we investigated the role of important vascular and inflammatory proteins, FKBPL and Gal-3, in preeclampsia using human plasma/placental samples and new 3D microfluidics model of placental tissue.

Methods: ELISA or Western blotting were utilised to determine FKBPL and Gal-3 concentrations/expression in plasma (n=17 controls; n=30 preeclampsia) or placental samples (n≥6 per group). Human umbilical vein endothelial cell (HUVEC) vascular formation, and remodelling by trophoblast cells (ACH-3Ps), were characterised using 3D microfluidics chips, exposed to inflammatory tumour necrosis factor (TNF)-α. Immunofluorescent staining, Western blotting and ELISA were used to determine the secretion/expression of FKBPL and Gal-3 in the 3D microfluidics placental model.

Results and discussion: FKBPL and Gal-3 protein levels were upregulated in plasma (FKBPL; $p<0.0001$, Gal-3; $p<0.05$) and placental (FKBPL; $p<0.05$, Gal-3; $p<0.05$) preeclampsia samples compared to controls. Inflammation in 3D vascularised microfluidics placental models also led to an increase in FKBPL and Gal-3 protein expressions (FKBPL; $p<0.05$, Gal-3; $p<0.05$), in conjunction with changes in vascular pattern (branching pattern) and reduced vasculo-genesis potential (CD31; $p<0.005$).

Conclusions: The 3D human placenta microfluidic model can recapitulate human preeclampsia demonstrating upregulation of FKBPL and Gal-3, which is indicative of restricted angiogenesis with potentially a key role in inappropriate placental development and vascular dysfunction in preeclampsia

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Luteal phase progesterone signalling impacts timing of parturition in mice via a $CD4^+$ T cell dependent mechanism

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The maternal immune response is central to the timing of parturition and labour, but the underpinning mechanisms are ill-defined. Increasingly, evidence points to the quality of immune adaptation in early pregnancy as a driver of pregnancy tolerance and protection from late gestation adverse outcomes. We recently demonstrated luteal phase progesterone (P4) signalling is a central regulator of the maternal $CD4^+$ T cell response in pregnancy. In a model of T cell dysfunction caused by impaired luteal phase P4 signalling, low-dose P4 antagonist RU486 (1 mg/kg) administered on 1.5 and 3.5 dpc led to poor pregnancy viability and fetal growth restriction. Transferring regulatory T (Treg) cells to RU486-treated mice showed that P4-induced Treg cells are necessary for healthy pregnancy and fetal growth. Unexpectedly, a parturition defect was also revealed, as RU486-treated dams delivered ~24 hours later than controls ($P<0.0001$; 18.8 vs 19.7 dpc) and failed to undergo serum P4 decline at 18.5 dpc ($P<0.05$ vs control), raising the question of whether T cells induced by P4 are involved in regulating parturition. Here, we investigated this by adoptively transferring $CD4^+CD25^+$ Treg cells or $CD4^+CD25^-$ T conventional (Tconv) cells into RU486-treated mice on 3.5 dpc. Interestingly, transfer of Tconv cells (P=NS vs control), but not Treg cells ($P<0.05$ vs control), restored the timing of birth following P4 signalling disruption. Furthermore, there was a strong inverse correlation ($r= -0.81$) between plasma P4 concentration on 18.5 dpc and number of viable fetuses, suggesting that fetal antigen-driven T cell responses might promote luteal regression preceding parturition. These data demonstrate that a distinct subset of $CD4^+$ T cells mediates events required for on-time parturition, and generation of this cell population depends on sufficient luteal phase P4 at the outset of pregnancy. These findings provide insight relevant to the pathophysiology of prolonged pregnancy and stillbirth risk in women.

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Gestational diabetes mellitus: more than just maternal obesity

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Gestational diabetes mellitus (GDM), a form of diabetes that occurs during pregnancy, impacts the mother and her baby perinatally and poses an increased risk of lifelong chronic disease for both[1]. The rate of GDM in Australia has tripled from 5.4% to 16.1% over the past decade[2].

Etiology of GDM is mostly related to obesity, maternal age and ethnicity[3], yet the rise in GDM is seen across all groups and the current trajectory began before the 2014 change in World Health Organisation diagnostic criteria. Recent evidence shows an association of folic acid (FA) with insulin resistance and GDM[4-6]. Interestingly, the Australian government implemented FA food fortification in September 2009; the rise in GDM incidence soon followed. Despite shared etiology, type 2 diabetes has not significantly changed in the past decade. We have therefore turned to the placenta, an organ unique to pregnancy and an essential regulator of maternal glucose tolerance, insulin sensitivity and glucose transport to the fetus. Placenta starts to secrete hormones early in normal gestation into maternal circulation to stimulate maternal beta cell expansion and insulin resistance and ensure adequate glucose transport to the fetus[7]. Our preliminary data from two pregnancy cohorts that recruited women prior to and post FA fortification mandate (SCOPE n=1164, 4.2% GDM; STOP n=1300, 15.2% GDM) shows circulating placental hormones at 12-16 weeks' gestation are substantially different between the cohorts. Mean maternal red cell folate post fortification is also elevated (SCOPE 619.0±15.6 nmol/L; STOP 1442.8±321.1nmol/L), levels exceeding normal RCPA reference range.

These data suggest altered placental endocrine responses to elevated folate should be investigated as a potential mechanism driving the GDM rise. In order to improve short and long-term health of more than 500,000 mothers and babies who are predicted to be diagnosed with GDM over the next decade, further research is urgently required.

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Primary human trophoblast expression of the renin-angiotensin system is sensitive to hyperglycaemia

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Gestational diabetes mellitus (GDM) is one of the most prevalent pregnancy complications in Australia and is associated with immediate and long-term morbidity for mothers and their babies. The placenta contributes to GDM pathogenesis by secreting factors that induce maternal insulin resistance, resulting in hyperglycaemia. The placental renin-angiotensin system (RAS) is a critical regulator of placental development however it is yet to be characterised in GDM. In non-pregnant type II diabetics, RAS activity is upregulated and mediates hyperglycaemia-induced tissue injury. Thus, we propose that the placental RAS is also dysregulated by hyperglycaemia in GDM, impacting placental structure and function.

Primary human trophoblast cells were isolated from placentae of women with uncomplicated pregnancies delivering by caesarean section at term. Trophoblast cells were cultured in normoglycaemic conditions [5mM glucose] for the initial 24h and subsequently cultured in normoglycaemic or hyperglycaemic conditions [25mM glucose] for an additional 48h (n=5 placentae in triplicate). The mRNA expression of angiotensinogen (*AGT*), angiotensin converting enzyme (*ACE*), prorenin receptor (*ATP6AP2*), angiotensin II type 1 receptor (*AGTR*1), and *ACE2* was measured by qPCR. ATP6AP2 and ACE2 protein levels were also determined by Western Blot.

Exposure to hyperglycaemia significantly reduced the mRNA expression of *ACE* (p=0.035) and *ACE2* (p=0.008), and tended to reduce the expression of *AGT* (p=0.077) and *AGTR1* (p=0.079). The mRNA expression of *ATP6AP2* was unchanged (p=0.600). ACE2 protein levels were not reduced by hyperglycaemia (p=0.149). Interestingly, despite no change in *ATP6AP2* mRNA expression, protein levels of ATP6AP2 tended to decrease in hyperglycaemic conditions (p=0.061). Protein levels of additional RAS components are under investigation.

The expression of RAS components in primary human trophoblast cells is sensitive to hyperglycaemia *in vitro*. In cases of GDM where hyperglycaemia is poorly managed, this may supress placental RAS signalling via trophoblast cells and negatively impact placental and potentially fetal growth.

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Reduced uterine natural killer cell abundance and impaired spiral artery remodeling in mice performing intensive exercise before and during pregnancy

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Introduction: Regulatory T (Treg) cells, a subset of anti-inflammatory immune cells, are essential for maternal immune tolerance and suppressing inflammation. Many women with preeclampsia have fewer Treg cells and preeclampsia is associated with impaired spiral artery remodeling.

In mice, Treg cells facilitate uterine artery function and spiral artery remodeling during early placental development. Exercise can increase Treg cell numbers in non-pregnant mice and enhance placental vascular volume in pregnant women. We therefore hypothesized that exercise before and during murine pregnancy would enhance Treg cell proportion and uterine natural killer (NK) cell abundance, leading to improved vascular adaptations and pregnancy outcomes.

Methods: Female CBA/J mice were exercised for 6 weeks prior to and throughout pregnancy on a treadmill (5 days/week, 10 m/min, 30 min/day). Control mice remained sedentary. On day 10.5 post coitum (pc), Treg cells in the uterine-draining lymph nodes (udLN) were assessed using flow cytometry, and uterine artery function, decidual spiral artery remodeling and uNK cell abundance were also analysed. On day 18.5 pc, fetal and placental weights were assessed.

Results: Intensive running exercise before and during pregnancy increased the proportion of Treg cells by 20%, along with a 35% increase in expression of the proliferation marker Ki67 (both $p<0.05$) at day 10.5 pc. Uterine artery haemodynamics were unchanged. Unexpectedly, decidual spiral artery remodeling was impaired and uNK cell abundance was reduced. At the end of pregnancy, fetal weight and the fetal:placental weight ratio were reduced by 25% and 28% respectively (both $p<0.05$) in exercised compared to sedentary dams.

Conclusion: Continued intensive running exercise before and during pregnancy increased Treg cell abundance, but impaired spiral artery remodeling and fetal growth. Ongoing experiments will assess whether a moderate exercise regime can obtain immune benefits without imposing excessive energy demands on the mother that may impair fetal outcomes after intensive exercise.

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Temporal expression of inflammasomes is increased in human placentae affected by fetal growth restriction (FGR), and in three inflammation-induced models of FGR: a murine model *in vivo*; human placental organoids and cultured BeWo cells *in vitro*.

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Background: Fetal growth restriction (FGR) is defined as a birth weight of <10th centile for gestational age, with severe FGR (<3rd centile) contributing significantly to stillbirth and iatrogenic preterm birth. The primary cause of FGR is placental insufficiency. The human placenta is immunologically active through trophoblasts, which generate specific and diverse innate-immune responses through the expression of multimeric self-assembling protein complexes, called inflammasomes. However, changes in expression levels of inflammasomes in the placentae of FGR are poorly understood.

Hypothesis: Placental inflammasome component expression is increased in human FGR tissues, in an inflammation-induced murine pregnancy *in vivo,* in an *in vitro* human placental organoid model system, and in the trophoblast-derived cell line model, BeWo.

Methods: Human placentae from third trimester idiopathic FGR (n=25) and gestation-matched uncomplicated control pregnancies (n=25, 28-40 weeks of gestation) were collected to determine the expression of the inflammasome components using a Fluidigm Biomark™ array, with independent validation by real-time PCR. Candidate inflammasome component expression was investigated in placentae following lipopolysaccharide treatment to induce inflammation in 1) a murine model *in vivo,* 2) an *in vitro* cultured 3-D self-organising human placental organoids generated from 6-9 weeks of gestation, and 3) in cultured BeWos.

Results: Placental mRNA expression for the inflammasome components *NLRP3*, *NLRC5*, *CASP1*, *NFκB1*, *IFNγ, IL-1β* and *IL-6*, was significantly increased relative to *18S rRNA*, while the anti-inflammatory cytokine, *IL-10* mRNA expression was significantly decreased in human FGR compared with control pregnancies ($p<0.001$). *NLRP3* correlated with gestational age in human FGR ($r=0.558$, $p=0.004$). NLRP3 protein expression was increased in LPS-induced inflammation in the murine placentae, *in vitro* models of human placental organoid system and BeWos.

Conclusion: Placental expression of inflammasomes components was associated with increased pro-inflammatory cytokine expression in FGR. Further functional analysis may identify inflammasomes as potential biomarkers of pregnancies at risk of developing FGR.

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Comparison of the proteomic composition of pregnant and non-pregnant mare plasma: identification of early pregnancy biomarkers

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Diagnosis of early pregnancy in mares is an important component of equine breeding practice, as early embryo loss is relatively common and incurs a substantial economic loss. This is compounded by a short breeding season, placing pressure on breeders to achieve pregnancies early. Furthermore, unlike in other domesticated livestock species, a precise signal or mechanism for maternal recognition of pregnancy has not yet been elucidated in horses. Clinical detection of mare pregnancy typically occurs around day 14 using trans-rectal ultrasonography. As a prelude to the development of an on-farm robust early pregnancy test,

we are undertaking proteomic analysis to compare the plasma profiles of pregnant (7P) and non-pregnant (7NP) mares at day 7 following ovulation to identify pregnancy-induced protein biomarkers. Using a batch-mode approach, we have sequenced 66 plasma samples from both 7P and 7NP mares using an Exploris 480 mass spectrometer in an unbiased fashion. Using our established bioinformatical pipelines, we have identified a plasma protein profile of 234 proteins with 13 of these displaying significantly different abundance between 7P and 7NP. Amongst these changes was serpin A6, a member of the serine proteinase inhibitor (serpin) plasma proteins, which was significantly increased in 7P plasma. Members of the serpin family have been identified as being synthesised by the uterus in many species, and serpin A6 is the principal transport protein for cortisol and progesterone, thus supporting a role for this protein in early pregnancy. Other differentially abundant proteins detected in this study may be important in the immunological recognition of pregnancy such as Immunoglobulin lambda light chain variable region, Alpha 2 macroglobulin and Complement C8 gamma chain etc. Overall, this study is providing an important platform to establish a panel of protein biomarkers for the confident detection of early pregnancy in the mare.

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Polycomb-dependent epigenetic programming in the oocyte impacts placentation and pregnancy in the next generation

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Epigenetic modifications are essential in regulating embryonic and placental development. However, the molecular basis through which epigenetic information is inherited from parents to offspring and the impacts this information has on placentation and pregnancy remains poorly understood.

Embryonic Ectoderm Development (EED) is an essential component of Polycomb Repressive Complex 2 (PRC2), a highly conserved epigenetic regulator required in oocytes for offspring growth and development. PRC2 catalyses tri-methylation of histone 3 lysine 27 (H3K27me3), thereby repressing developmental genes in multiple tissues. PRC2 is an important epigenetic modifier of placentation, with disruption of its subunits linked to placenta-associated disorders.

To determine the role of PRC2 in programming inherited impacts on placentation, we deleted EED only in growing mouse oocytes and analysed placental and pregnancy outcomes. Oocytes lacking EED had severely depleted H3K27me3 and widespread gene derepression, including *Plac1*, an X-linked gene essential for placental and embryonic development (n=4-6, P=6.7E-13; FDR=3.19E-09). Moreover, embryonic offspring from oocytes lacking EED initially demonstrated significant embryonic growth restriction (n=15-38, p<0.0001), followed by catch-up growth. Placentas from these offspring were initially similar in weight to genetically identical but epigenetically different controls but became significantly larger in mid-late gestation (n=32-68, p<0.0001). Histological analyses of E17.5 placenta from EED deficient oocytes showed an expanded junctional zone with abnormal projections of spongiotrophoblast into the labyrinth. Effects on pregnancy were also evident through decreased litter size (n=12-29, p<0.0001) and increased gestational length in litters derived from oocytes lacking EED (n=7-13, p=0.0068). Current RNA sequencing is expected to identify pathways involved, providing insight into the mechanisms regulated by EED-dependent maternal inheritance.

Together, these data provide evidence that EED-dependent epigenetic programming in the oocyte plays an essential role in regulating placental development and pregnancy. Further defining this, and similar mechanisms, will provide insight into how altered epigenetic inheritance affects placental function and offspring development.

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Nicotine exposure reduces uptake of transthyretin-thyroxine by placental trophoblasts

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Publish consent withheld

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A preliminary investigation into factors influencing the success of ovine artificial insemination

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Artificial insemination (AI) programs play a key role in facilitating rapid genetic and production gains in the sheep industry. However, variable rates of success remain a concern and can be a barrier for adoption. The degree to which various male and female factors influence, and ultimately predict fertility following AI remains unknown, largely due to a lack of large-scale industry data sets for analysis. As such, a preliminary investigation of the effect of several factors which may contribute to the variation in the success of ovine AI was made using data compiled from three separate industry AI programs (N = 3663 ewes). Briefly, sire (n=31), time of AI following progesterone pessary removal (48-57h), uterine tone (scored 1-5) and visual intra-abdominal fat score (scored 1-5) of ewes were recorded at AI and pregnancy rate of each ewe subsequently determined by ultrasound (>50 days post AI). Multivariate regression analysis revealed the likelihood of pregnancy varied substantially with sire ($P<0.001$), with fertility per sire ranging from 47.2% to 78.3%. AI time post pessary removal, uterine tone and visual intra-abdominal fat score fell short of significance within the multivariate model. These preliminary findings highlight the variability in sheep AI results that can be attributed to sire. Future research involves an industry wide assessment of the aforementioned factors to increase the size of the dataset for analysis and experimental power. Further, advanced in vitro assessment of semen used for AI will be undertaken to understand the reasons behind inter-male variation in fertility and to build a model for the accurate prediction of AI success.

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Prenatal BPA programming of muscle transcriptome in female sheep

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Bisphenol-A (BPA) is a ubiquitously present endocrine disrupting chemical, and prenatal exposure produces long-term metabolic dysfunction in the offspring. In female sheep, prenatal BPA induces peripheral insulin resistance with tissue-specific changes in negative mediators of insulin resistance. In skeletal muscle this is manifested as increased oxidative stress markers, triglyceride accumulation, and reduced antioxidant gene expression. To identify changes in gene expression and potential biomarkers of metabolic disruptions, RNA expression was compared in skeletal muscle from prenatal BPA-treated (daily subcutaneous injections of 0.5mg/kg/day BPA in corn oil from days 30 and 90 of gestation (term 147 days)) and control females (corn oil injections for same duration). Prenatal BPA dysregulated 112 genes (32 downregulated and 80 upregulated) at false discovery rate adjusted p value (FDR) <0.05 and absolute $\log_2$ fold change>0.5. Gene enrichment analysis showed that prenatal BPA dysregulated 194 gene pathways at FDR<0.01, including pathways related to RNA biosynthetic process, immune function, and collagen synthesis. Orthogonal Projections to Latent Structures Discriminant Analysis identified potential biomarkers of prenatal BPA exposure including downregulation of calpastatin (*CAST*), nitric oxide synthase 1 (*NOS1*), deoxyribose-phosphate aldolase (*LOC101105149*), serum amyloid A protein (*LOC101120204*) and upregulation of Fas apoptotic inhibitory molecule (*FAIM*), fatty acid synthase (*FASN*), DENN domain containing 2B (*ST5*), family with sequence similarity 71 member A (*FAM71A*), and arylsulfatase D (*LOC101116024*). Downregulation of the antioxidant NOS1 and upregulation of the lipid synthesis gene FASN are in line with the increased oxidative stress and lipid accumulation in the muscle from prenatal BPA-treated sheep. Further investigation of the mechanisms by which these biomarkers play a role in the overall functional phenotype will help understand the complex effects of prenatal BPA exposure on metabolism.

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HtrA4 is expressed only in the placenta of primates and plays an essential role in trophoblast invasion

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Objectives: HtrA4 (high temperature requirement factor A4) belongs to a family of serine proteases that are evolutionarily conserved among all organisms. In general, these proteases play important roles in many cellular processes such as growth, unfolded protein stress response and programmed cell death. In the human, there are four HtrA homologs, whilst HtrA1-3 are widely expressed, HtrA4 expression has so far been detected only in the placenta and its aberrant expression is associated with pregnancy-related diseases. This study aimed to determine whether the expression of HtrA4 is restricted to the placenta of primates and to investigate the potential role of HtrA4 in trophoblast biology.

Methods: Various databases were searched to bioinformatically analyze the expression of HtrA4 in diverse tissues in the human, rhesus monkey and mouse. Comparative analysis was then conducted across these species to determine whether HtrA4 expression is primate specific. The results were then experimentally confirmed by RT-PCR analysis of HtrA4 mRNA in all 3 species, and by immunohistochemical analysis of HtrA4 protein in human and rhesus monkey tissues. Next, the HtrA4 gene was silenced by CRISPR in human trophoblast cell line BeWo and the impact on trophoblast invasion and migration was examined using xCELLIgence.

Results: In both the human and rhesus monkey, HtrA4 mRNA was abundantly expressed only in the placenta, whereas in the mouse, HtrA4 was not expressed in any tissues including the placenta. In the human and rhesus monkey placentas, HtrA4 was highly expressed in the syncytiotrophoblast and extravillous trophoblasts from early stages of pregnancy. HtrA4 silencing in BeWo significantly inhibited cell invasion but did not affect migration.

Conclusions: HtrA4 expression is restricted to the placenta of primates, and it plays an important role in trophoblast invasion which is critical for primate placental development and function.

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Leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) and its ligands are reduced in preeclamptic placentas.

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Lgr5 is an established stem cell marker and Wnt signalling pathway member with abundance in high surface area tissues, namely the colon and lung. The placenta is a complex organ of high surface area and significant branching dedicated to maternal-fetal exchange. Lgr5 has not been explored in human placental development, nor in placental diseases including preeclampsia. This study sought to characterise Lgr5 in preeclampsia and assess its role in isolated human (cyto)trophoblast (placental) stem cells (hTSCs).

Lgr5 was measured in placentas of patients presenting with early-onset (<34-weeks gestation, n=81 vs n=19 controls) and late-onset preeclampsia (≥34-weeks, n=20 vs n=61 controls). In both cohorts' preeclamptic placental samples, Lgr5 mRNA was significantly reduced ($p<0.0001$, $p=0.0046$ respectively). Western blot of preeclamptic vs preterm control placental lysates confirmed this. Immunohistochemistry localised Lgr5's expression to both cytotrophoblast (progenitor) and syncytiotrophoblast (terminally differentiated) populations in human placenta. In contrast to primary first trimester placental cells, the hTSCs have very low expression of Lgr5. Thus, we over-expressed Lgr5 to assess its functional role in proliferation and differentiation. Preliminary data suggests increasing cytotrophoblast Lgr5 alone does not alter cytotrophoblast proliferation or differentiation to syncytiotrophoblasts or extra-villous cytotrophoblasts.

Since Lgr5-mediated signalling requires binding of its ligands, R-spondins 1, -3 and -4; their expression was investigated in control vs preeclamptic placentas and maternal plasma. R-spondins 1 and -4 were reduced in pre-term preeclamptic placentas ($p=0.0005$, $p=0.0003$ respectively), while R-spondin 3 was unaltered. In maternal plasma, R-spondin 4 was undetectable yet R-spondins 1 and -3 were elevated in preeclampsia. Additional functional studies modulating both Lgr5 and its ligands are currently underway.

This study demonstrates placental Lgr5 and its ligands R-spondins 1 and -4 are reduced in preeclampsia. Their effects on placental cell function and placental development are currently being explored in detail to elucidate potential roles in preeclampsia pathogenesis.

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Long-term exposure of mice to 890 ppm atmospheric CO_2 increases birth weight, modifies growth trajectories, and alters behaviour in young adulthood

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Atmospheric carbon dioxide (CO_2) levels are currently at 418 parts per million (ppm), and by 2100 may exceed 900 ppm. The biological effects of lifetime exposure to CO_2 at these levels is unknown. We have previously shown that mouse lung function is altered by long-term exposure to 890 ppm CO_2. Here, we assess the effects of exposure on pregnancy parameters, postnatal growth, and broader systemic responses to this exposure. Mice were exposed to either 460 ppm or 890 ppm from preconception to 3 months of age, and assessed for effects on maternal weight gain, litter size, sex ratio, birth weight, and postnatal growth. As young adults, renal and osteological parameters, locomotion, memory, learning, and anxiety-like behaviours were assessed. Exposure to 890 ppm CO_2 increased birthweight by 15% ($p<0.05$) in comparison to controls but did not alter any other pregnancy outcomes. From postnatal day 7 onwards, there were no differences in body weight until after weaning when female body weight was decreased by exposure to 890 ppm CO_2. As young adults, life-long exposure to 890 ppm CO_2 resulted in reduced engagement in memory/learning tasks, and hyperactivity in both sexes in comparison to controls. There were no clear anxiety, learning, or memory changes. Renal and osteological parameters were minimally affected. Overall, this study shows that exposure of mice to 890 ppm CO_2 from preconception to young adulthood increases birthweight, alters growth and some behaviours, with limited evidence of compensatory changes in acid-base balance. The increased birth weight due to elevated CO_2 exposure during conception and pregnancy were unexpected. The drivers of this change may be due to alterations in maternal adaptations to CO_2 and/or placental function. These findings highlight the potential for a direct effect of increased atmospheric CO_2 on mammalian health outcomes.

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MEK1/2 regulates male germline development independent of FGF signalling

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Mammalian male or female development depends on testis or ovary formation, involving sex-specific transcription and signalling in embryonic gonads. Disrupted testis and germline development is strongly associated with testis cancer in humans. In mice, *Sry* and *Sox9* promote testis cord formation, committing the fetus to male development. SRY and SOX9 induce Sertoli cell *Fgf9* expression, essential for testis development and is thought to promote male germline differentiation. As FGFs signal through Mitogen-Activated Protein Kinase (MAPK) in other tissues, we explored whether FGF9 regulates male germline development through MAPK by inhibiting FGF or MEK1/2 signalling.

Embryonic day (E)11.5-12.5 Oct4GFP transgenic mouse testes were cultured with FGF receptor (FGFRi) or MEK1/2 (MEKi) inhibitors for 24-96h. Impacts on testis cords and Sertoli, somatic and germ cell development were determined using immunofluorescence, flow cytometry and RNA sequencing.

E12.5+72h FGFRi or MEKi culture reduced Sertoli cell proliferation and disrupted testis cord formation; characterised by mis-location of Sertoli cells throughout testis cords and germ cells mis-localised outside testis cords. Assessment of DPPA4 and DNMT3L expression and mitotic arrest indicated that male germline differentiation was prevented by MEKi. Moreover, some germ cells expressed female germline markers but did not enter meiosis. Meanwhile, E12.5+72h FGFRi culture did not affect male germline differentiation, though E11.5+96h FGFRi culture had a slight, but insignificant, impact on mitotic arrest. To identify developmental pathways disrupted by FGFRi or MEKi, global transcriptional regulation is currently being assessed in germ and somatic cells.

Together, our data indicate essential roles for MEK1/2 signalling in male germline development, but a surprisingly limited role for FGF signalling. While FGF9 may partially promote male germline development, our data strongly indicate that additional ligands acting through MEK1/2 play a significant role. Our work highlights a need for further understanding of mechanisms underlying gonad development and testis cancer in humans.

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BMP signalling facilitates meiotic progression in mouse fetal germ cells, *in vivo*

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Essential to sexual reproduction is the generation of haploid gametes from diploid germ cells through meiosis – a specialised reductive form of cell division that occurs exclusively in the germline. Despite its importance, the molecular mechanisms underlying the transition from mitosis to meiosis remains poorly understood. Germ cells initiate meiosis during fetal life in females and at puberty in males. This temporal difference is widely believed to be dependent solely on the gonadal environment established by the somatic cellular population, not the chromosome constitution of the germ cells (XX vs XY). In females, the extrinsic signalling molecule retinoic acid (RA) activates a meiotic gatekeeper, *Stra8*, which is indispensable for meiosis in both sexes. Nonetheless, other signalling factors such as bone morphogenetic proteins (BMP) are also likely to be involved to ensure germ cells embark on meiosis at the right time.

Recent work of Miyauchi *et al.* suggested BMP may be required in addition to RA to induce the female fate in primordial germ cell-like cells *in vitro*. To investigate whether and how BMP signalling is required *in vivo*, we assessed a mouse model deficient for BMP receptor 1A and showed that germ cell-specific loss of BMP signalling does not impact on *Stra8* expression, but does delay meiotic onset and progression in female fetal germ cells. More experiments are currently underway to identify potential downstream targets of BMP in fetal ovaries, and to thoroughly unveil the molecular mechanisms underlying a timely meiotic transition and efficient progression in mouse germ cells.

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Androgen receptor signaling in GABA neurons is not necessary for the development of PCOS traits in a peripubertal PCOS mouse model

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Polycystic ovary syndrome (PCOS) is the most common endocrine disorder affecting reproductively aged women. Hyperandrogenism is a key characteristic of PCOS and many women with PCOS also experience luteinizing hormone hypersecretion. Although the origins of PCOS are unknown, evidence in animal models strongly implicates androgen signalling in the brain. Gamma-aminobutyric acid (GABA) expressing neurons have a role in stimulating gonadotrophin releasing hormone (GnRH) secretion. Circuit remodeling of GABA neurons has been observed in hyperandrogenic PCOS animal models, with studies reporting an increase in GABAergic innervation and activation of GnRH neurons. This suggests that AR-mediated disruption of GnRH neuron activity may occur primarily via GABA neurons.

To investigate the role of androgen actions in GABAergic neurons in PCOS pathogenesis, we combined a dihydrotestosterone (DHT)-induced PCOS mouse model with a GABA specific AR knock out (GABARKO) mouse model and assessed the development of PCOS-like traits after 12 weeks of DHT exposure.

DHT exposure in both WT and GABARKO females induced key reproductive and metabolic PCOS traits. DHT exposed WT and GABARKO mice both exhibited anovulation and acyclicity. Moreover, DHT treatment, but not genotype, had a significant main effect on total body weight and retroperitoneal fat pad weight in WT and GABARKO mice (2-way ANOVA $P<0.05$). DHT treatment had significant main effects on adipocyte size in retroperitoneal fat ($P<0.05$), with no significant difference observed between GABARKO and WT mice. Lastly, DHT exposure had significant main effects over blood glucose IAUC ($P<0.05$), but no difference was observed between GABARKO and WT PCOS mice.

Overall, these data show that impeding AR signaling in GABA neurons does not change the development of PCOS-like traits in mice chronically exposed to DHT. Therefore, these findings suggest that primary androgen actions in the female brain driving the pathogenesis of PCOS are likely mediated via non-GABAergic neurons or non-neuronal cells.

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A proteomic approach to investigating oxidative stress in human spermatozoa – lipid aldehyde induced protein modifications and therapeutic potential

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Infertility is a growing health burden, with an estimated 80 million individuals experiencing the impact of this disease globally. This is particularly concerning as there is now compelling evidence supporting the paradigm that male infertility is augural of chronic illnesses such as cardiovascular disease, diabetes, and cancer. Our previous work has demonstrated that oxidative stress is a major underlying aetiology of male infertility as the oxidative products of lipid peroxidation can disrupt sperm-egg interaction. Moreover, we have developed a lipid-based strategy to protect human sperm function during oxidative stress through the inhibition of arachidonate 15-lipoxygenase (ALOX15), a catalyst of lipid oxidation and reactive carbonyl species production (including 4-hydroxynonenal; 4HNE and malondialdehyde; MDA). Despite these steps forward, we do not know the full inventory of sperm proteins that are susceptible to oxidative damage or whether ALOX15 inhibition can prevent these oxidative modifications en masse.

To characterise the oxidation-vulnerable human sperm proteome we exploited high-resolution tandem mass spectrometry to quantify post translational modifications (PTMs) in human spermatozoa following an oxidative challenge induced by 50 µM 4HNE. This strategy successfully identified 1,999 proteins, and 4,729 site specific PTMs. 4HNE exposure induced significant upregulation in lipid peroxidation-related PTMs, with modification events (featuring 4HNE, MDA and oxidation) identified for 40 proteins [2-fold change, $p \leq 0.05$]. These modified proteins included our previously validated targets for oxidative damage, heat shock protein A2 and A-kinase anchoring protein 4, as well as numerous novel oxidation targets. Moreover, the modified proteins identified are known to play key roles in protein homeostasis (45%; e.g. heat shock protein 90), cell metabolism (55%; e.g. succinate dehydrogenase) and fertilization (55%; e.g. acrosin-binding protein). Through these findings we are now well positioned to investigate the therapeutic efficacy of lipoxygenase inhibition at a functional proteomic level. This will assist in the prevention of oxidative stress-induced male infertility.

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Vitamin E partially rescues reproductive function in male *Drosophila melanogaster* exposed to heat

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Prolonged exposure to high temperature increases oxidative stress in the male reproductive tract, reducing sperm quality and contribute to male infertility. Vitamin E, a potent antioxidant, is used therapeutically to counter many pathologies that are related to oxidative stress. It is not known whether vitamin E can help to preserve fertility during exposure to heat stress. Hence, this study was designed to investigate whether dietary supplementation with vitamin E would preserve male reproductive capacity in *Drosophila melanogaster* during exposure to heat stress. For 15 days, adult male flies were exposed to either room temperature continuously (RT; 25°C, n = 96) or to heat stress for eight hours each day, and 25°C for the other 16 hours per day (HS; 34°C, n = 96) and fed one of four levels of vitamin E in the food (0, 10, 100, or 1,000 µg/mL). After treatment, half of the flies from each treatment group (n = 12) were placed individually into vials with one virgin female. Their reproductive capacity was assessed by counting the number of eggs and the number of offspring in each vial. The remaining flies from each treatment group (n = 12) were used to evaluate the average size of the seminal vesicles and sperm DNA fragmentation. Heat stress reduced the number of eggs, offspring, and seminal vesicle size ($P<0.05$), and the effect was partially rescued by vitamin E supplementation ($P < 0.05$). Compared to heat stressed males without vitamin E, those supplemented with 100 µg/mL vitamin E during heat stress had a lower percentage of sperm with DNA fragmentation. Overall, we conclude that vitamin E can help to maintain the reproductive capacity of male *D melanogaster* during exposure to heat stress. Whether mammals, including humans, similarly benefit from antioxidant supplementation during exposure to higher temperatures remains to be established.

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Can paternal H3K4me3 transmission in sperm affect imprinting status of paternally-expressed genes after fertilisation?

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During the establishment of genomic imprinting, only one copy of the two parental alleles is activated, regulated by a differential epigenetic status of the mother's or father's chromosomes. While DNA methylation and suppressive histone modifications established during gametogenesis play a crucial role in supressing imprinted genes on the inactive allele, how and when the expressed allele gains its active status is not clear. It is possible that active chromatin status could be inherited from the active histone marks in gametes. However, transmission of paternal active histone marks would not occur if histones are replaced with protamine by the histone-to-protamine transition that occurs in spermatozoa in the final stages of maturation. In this study, we

asked whether active histone marks remain in sperm throughout the histone-to-protamine transition and whether they remain active after fertilisation. We analysed published data to assess the active status of paternally-expressed genes. Mouse sperm retained the active histone mark, histone-3 lysine-4 tri-methylation (H3K4me3) at more than half of known paternally-expressed genes, regardless of their association with DNA methylation. The transmitted paternal histone status was retained during early embryogenesis, suggesting that the paternal H3K4me3 transmission defined active status of the paternally-expressed genes. Using reciprocal cross data, novel paternally-expressed genes during zygotic genome activation were identified. The majority of the identified paternally-expressed genes retained H3K4me3 in sperm. However, differential epigenetic status at some of the novel imprinted genes was reprogrammed as embryogenesis proceeds. The loss of differential epigenetic status between the alleles coincided with their loss of imprinted gene expression during pre-implantation development. The retention of H3K4me3 through the histone-to-protamine transition may be a mechanism for transmitting active chromatin status of paternally-expressed genes.

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Biochemical changes in ram spermatozoa after in vitro exposure to common herbal supplements

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Complimentary and alternative medicines are used frequently, despite a lack of regulation and research into their safety and efficacy. Supplements marketed towards 'boosting fertility' are popular, but there is little research into their effects on spermatozoa. Previous studies looking at supplementation of sperm focus on sperm motility rather than biochemical changes, and there is little research regarding potential toxicity in sperm.

The function and metabolism of ram spermatozoa, incubated in media containing *Lepidium meyenii* (maca root) were investigated. At 0.5, 3 and 6 hours post-exposure, computer-assisted sperm analysis (CASA) and flow cytometry were used to assess motility, viability, acrosome reaction, membrane lipid disorder, mitochondrial superoxide production, intracellular reactive oxygen species (ROS) and DNA fragmentation. Treatment with maca induced acrosome reaction in treated cells ($p < 0.001$), but there was no difference in viability ($p > 0.05$). There was an increase in mitochondrial superoxide production across all treatments and time points ($p < 0.001$), and maca promoted membrane lipid disorder across all treatments ($p < 0.001$).

The promotion of premature acrosome reaction in absence of an ovum may impact fertility of the male. It is posited that this may be driven by the oestrogenic activity of maca. Oestrogenic activity may also contribute to the higher membrane fluidity of treated sperm, as oestrogen can induce capacitation-like changes. Despite reported antioxidant activity of maca, the increase in mitochondrial superoxide production suggests increased oxidative stress in the presence of maca. Contrary to popular belief that these products enhance fertility, this research indicates that they should be used with caution due to possible negative effects on sperm, and lack of stringent safety data.

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New insights into sperm ultrastructure through enhanced scanning electron microscopy

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The ultrastructural analysis of spermatozoa morphology is critically important to understand male fertility and the aetiology of infertility, including evolutionary processes [1]. Modern scanning electron microscopy (SEM) provides visualization of external and internal (using an automated ultra-microtome or focused ion beam to serially remove thin layers from the surface of the resin embedded sample) with nanometre resolution. To utilize this possibility, the protocols of sample preparation should be revised to provide the ultimate preservation of the ultrastructural features.

We have developed and validated two protocols of sample preparation to examine of the external morphology of sperm (with preservation of the plasma membrane and with its removal) and two high-pressure freezing and freeze-substitution based protocols for examination of the internal morphology of sperm [2].

The developed advanced sample preparation protocols and visualization using a high-resolution FEG-SEM (ThermoFisher Elstar G4 operating at accelerating voltage of 2 kV) allow previously unappreciated insights into mouse sperm ultrastructure, including the identification of novel structures within the fibrous sheath and domain-specific interactions between the plasma membrane and exosome-like structures.

We believe this is the first example of three-dimensional ultrastructural analyses of the sperm using FIB-SEM tomography. This technique provides the full-volume reconstruction of cells and can dramatically improve the understanding of the structure-function relationship in sperm, including allowing accurate three-dimensional topology of relevance to sperm motility research and ultimately the understanding of sperm competition. We believe the developed protocols have the potential to accelerate discovery in the relationship between sperm structure and function, including the analysis of the consequences of genetic and environmental factors.

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Pseudogene RNA content of sperm, could they be playing a role in paternal transmission of obesity?

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The RNA content of sperm is implicated in the transmission of obesity from the paternal linage. Relative to their size mature sperm contain significant amounts of RNA including non-coding RNAs (ncRNAs), pseudogenes (processed/unprocessed) and protein coding mRNA, with sperm RNA content delivered to the oocyte at fertilisation. To date, only one paper has reported the variations in RNA content of sperm from obese men (1), focusing on small ncRNAs. Utilising bioinformatics pipelines and broad range RNA capture, we assessed the effects of obesity on human sperm RNA content, including those RNAs present/absent due to obesity.

RNA content was extracted using the Trizol method from purified motile sperm (swim up) from eight normal weight (BMI: 18-25kg/m2) and eight obese (BMI: >30kg/m2) Caucasian normospermic men (<45 years) undergoing infertility treatment due to female factor. Samples were processed with Bioo small RNA isolating kit (up to 150nt) and RNA-seq libraries sequenced with ~15 million reads. Raw data was analysed via two pipelines: standard bulk RNA-seq and the bcbio small RNA-seq. The limma-voom method identified differential gene expression.

The most abundant sperm RNA species were ribosomal RNA ~40%, mRNA ~25%, mitochondrial transfer RNAs ~9%, long ncRNAs ~7% and pseudogenes ~5%. 7452 RNAs in sperm were increased in abundance due to obesity (3106: 42% pseudogenes) and 160 decreased in abundance (33: 20% pseudogenes). 267 RNAs were unique to sperm from normal weight men (122: 46% pseudogenes) while only three RNAs were unique to sperm from obese men.

Pseudogenes contributed to the majority of differentially abundant sperm RNAs between normal weight and obese men. Previously presumed to lack function, evidence suggests that pseudogenes play important biological roles including initiating innate immune response, source of ncRNAs, DNA mediated regulation, protein transcription, and may also somewhat mediate the transmission of obesity from fathers to children.

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Causative mechanisms and functional correlates of MTT reduction in stallion sperm.

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MTT is a commonly used cell vitality probe which forms insoluble formazan deposits at cellular locations of intense oxidoreductase activity. While the majority of this activity occurs within the mitochondria, extra-mitochondrial sites of MTT reduction have been recognized in the spermatozoa of several mammalian species. Therefore, the aim of this study was to determine the major sites and causative mechanisms of MTT reduction in stallion spermatozoa.

Spermatozoa were collected from miniature pony stallions, and ejaculates were separated into high- and low-density fractions using discontinuous Percoll gradients. Cells were incubated with 0.5 mg/mL MTT for 1 h at 37 ^{o}C, before being scored for formazan deposition via microscopy. A number of reagents were also assessed for their impact on MTT reduction by incubation with spermatozoa for 20 min before MTT addition. These reagents included the NADPH oxidase (NOX5) inhibitors VAS2870 (10 µM) and zinc (0.5 mM). Additionally, spermatozoa were incubated under capacitating conditions (0.72 mg/mL pentoxifylline, 0.6 mg/mL methyl-beta-cyclodextrin and 2.45 mg/mL dibutyryl-cyclic AMP), before the addition of MTT.

Our results show that both mitochondrial and extra-mitochondrial MTT reduction (formazan deposition) was greater in high-density compared to low-density spermatozoa (mitochondrial: 83.7±1.6 vs 30±5.2%; $P \leq 0.001$; extra-mitochondrial: 20.8±2.5 vs 12.2±3.2; $P \leq 0.05$). NOX5 activity was implicated in extra-mitochondrial MTT reduction, with NOX5 inhibitors suppressing this activity (VAS2870: 6.6±1.9 vs 19.3±2.9%; $P \leq 0.01$; zinc: 11.7±4.4 vs 22.6±3.7; $P \leq 0.05$; for treated vs control respectively), without impacting sperm vitality. Furthermore, extra-mitochondrial formazan deposition was greater in capacitated compared to non-capacitated spermatozoa (20.4±1.7 vs 34.6±3.4; $P \leq 0.001$), suggesting that NOX5 generated ROS play a role in mediating the redox regulation of equine sperm capacitation.

In conclusion, MTT reduction patterns by stallion spermatozoa are reflective of a species dependent on OXPHOS and one exhibiting NOX5 activity, with extra-mitochondrial redox activity being reflective of sperm quality.

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SNAI2 is required for germ cell differentiation during early postnatal testis development

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Publish consent withheld

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Importins: Diverse roles in male fertility explored using new and old tools

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Regulated nucleocytoplasmic transport is central to gene expression changes that underpin cellular development and homeostasis, including in the testis, and proteins in the importin family are the predominant facilitators of cargo transport through the nuclear envelope. Earlier reports by us and others that documented cell-specific profiles of importin transcripts and proteins during spermatogenesis led us to hypothesize that importins facilitate developmental switches in the testis. Importins serve additional functions, both inside and outside the nucleus that include acting as subcellular scaffolding, mediating cellular stress responses, and controlling transcription. The importance of importin functions is further supported by multispecies studies in which importin gene knockouts showed they are variously essential for life and fertility. To expand knowledge of importin roles and their potential transport and non-transport functions in the testis, we combined new and old tools. This involved side-by-side analysis of published single cell RNAseq (scRNA-seq) data and immunohistochemistry on sections of developing and adult mouse testes. The single cell transcriptomes were delineated and compared to the intracellular and intercellular locations of importin proteins, in both germ and somatic cell populations. The importin beta 1 transcript and its encoded protein, IMPb1, required for classical alpha/beta transport of cargo proteins into the nucleus, are abundant in maturing somatic cells after they cease mitosis, whilst in maturing germ cells, levels decline in post-mitotic spermatocytes and are absent in elongated spermatids. This highlights differential reliance on classical transport in distinct cell types. The importin alpha proteins, IMPa2 and IMPa4, display distinct nuclear and cytoplasmic localization profiles as spermatogenesis progresses. These changes in transcript expression profiles and importin protein subcellular distribution spotlight changing roles during testis development. Prospective analyses of existing human testis scRNAseq data can also identify additional avenues for impactful investigations of these conserved and highly important proteins.

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Assessment of the functional consequences of seminal fluid extracellular vesicle interactions with human spermatozoa

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Seminal fluid contains one of the most abundant extracellular vesicle populations of any bodily fluid. Seminal fluid extracellular vesicles (SFEVs) are proposed to perform a variety of functions including impacting sperm motility, capacitation, and the ability to undergo an acrosome reaction. However, variations in SFEV isolation and culture methods have led to inconsistencies in published data and hence their specific functions remain controversial. Here, we explored the functional consequences of SFEV interaction with human spermatozoa. Seminal plasma was collected from healthy donors and SFEVs were isolated in accordance with the Minimal Information for Studies of Extracellular Vesicles 2018 guidelines. Sperm:SFEV interactions were assessed *in vitro* at pH 5 and 7, mimicking the physiological pH spermatozoa encounter within the vagina and cervix/uterus, respectively. Sperm:SFEV docking capacity was assessed over a 1 hour co-culture period with biotin-labelled SFEVs. The functional consequences of sperm:SFEV interactions was assessed across 5 hours by Computer Assisted Sperm Analysis (motility) and immunocytochemistry (capacitation and acrosome reaction). SFEVs were observed to dock with spermatozoa and deposit biotinylated protein cargo (pH 5= 70.8%, pH 7= 88.3%) within 1-minute post incubation, with most labelling detected in the sperm tail. Sperm:SFEV binding did not further increase across the time course, nor did it vary according to pH. We also found no evidence that SFEVs altered sperm motility, capacitation status or the acrosome reaction. These findings demonstrate that while SFEVs have the capacity to interact with spermatozoa and deposit their cargo, under our co-culture conditions this interaction did not regulate sperm functional parameters. Altogether, these data raise the prospect that SFEVs may have alternate roles in the female reproductive tract. Given that extracellular vesicles are critical immune modulators in other physiological contexts, our future studies will focus on exploring the impact of SFEVs on the immune environment within the female reproductive tract.

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Activin A modulates the pace of germ cell development at the onset of spermatogenesis

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Male infertility and testicular cancer are thought to result from disruptions to testis development *in utero*. Physiological perturbations during human pregnancy can feature high activin A levels, and this study investigates how elevated activin signalling affects the cells destined to form sperm. After birth, testicular germ cells resume proliferation and transform into either differentiating spermatogonia that initiate the first round of spermatogenesis, or into spermatogonial stem cells (SSCs) that maintain spermatogenesis in adults. We studied a mouse model with elevated activin A bioactivity (*Inha* KO; lacks the inhibin a subunit, a potent activin inhibitor), to determine whether this affected establishment of spermatogonia or SSCs.

Immunofluorescent analysis was used to score germ cell populations; spermatogonia (marked by SALL4+), nascent SSCs (GFRA1+) and proliferating cells (Ki67+) in *Inha* WT and KO mouse testis sections at P0, P3 and P6. We discovered that *Inha* KO testes at P0 have 50% fewer germ cells, indicating vulnerability to elevated activin A during fetal life. Of the remaining germ cells, a higher proportion were GFRA1+ and Ki67+, suggesting advanced development. At P6, when the SSC population is fully established, we observed a higher proportion of GFRA1+ cells in *Inha* KO testes indicating that elevated activin A favours SSC formation. RNAseq analysis of P0, P3, P6 whole testis samples confirmed transcripts associated with SSCs (*Gfra1*, *Id4*, *Etv5*, *Bcl6b*, *Chd4*) and differentiated spermatogonia (*Sohlh2*, *Dnmt1*) were elevated in *Inha* KO testes. This further indicates accelerated/altered spermatogonial development occurs in high activin conditions; whether this is indirect or direct is under investigation using cultured spermatogonia. These new data, showing systemic activin A levels determine the pace of germ cell development and stem cell establishment, suggest that human pregnancy conditions with elevated activin A can influence the male germline and consequently may affect adult fertility.

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The role of ATP6AP2 in male fertility

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ATP6AP2 has been shown to be a multi-functional protein, which is involved in a number of cellular pathways including WNT and MAPK signalling, protein sorting and folding, and receptor-mediated endocytosis and recycling. Many of these functions depend on its interaction with the vacuolar H^+-ATPase (V-ATPase). Due to its role in WNT signalling, we hypothesised that ATP6AP2 plays a role in ovarian development. To test this hypothesis, we deleted *Atp6ap2* specifically in gonadal somatic cells during fetal development using the *Nr5a1*-Cre mouse. Surprisingly, these mice appear to develop normally pre-birth, and were born at the expected Mendelian ratio. However, both males and females were infertile. At three months of age conditional *Atp6ap2*-deficient XY presented with significantly smaller testes caused by the loss of spermatogenic cells. Here we present our data to date regarding the role of ATP6AP2 in testicular somatic cells and how its loss results in male infertility.

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Pregnancy specific paracrine factors alter the flow-mediated response of uterine radial arteries

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Background: Sufficient oxygen and nutrient supply to the baby is facilitated by adapting the utero-placental vasculature. Inadequate adaptation can cause fetal growth restriction, which goes undetected in ~50% of cases before birth. Uterine radial arteries (RAs) have been identified as rate-limiting for placental blood supply, but adaptation mechanisms are insufficiently studied. This work aims to combine computational and experimental approaches to understand how paracrine factors facilitate RA remodelling.

Methods: A rat uterine-specific computational model was developed, informed by direct measurements and µCT-scans (n=3/group), and used to predict RA flow rates. RA responses to increasing pressure and flow were determined in virgin and late-pregnant (E19.5) Sprague-Dawley rats (n=6-10/group) by pressure myography. Virgin RAs (n=5-6/group) were pre-incubated with estrogen, progesterone, PlGF, and/or VEGF to examine their influence on flow-mediated reactivity. Nitric oxide involvement was assessed with L-NAME.

Results: Under pressure-only conditions, inner diameters across pressure steps were significantly larger in RAs from pregnant animals (p=0.04). Both groups developed myogenic tone ≥50mmHg (*in vivo* pressure), with the response more pronounced in RAs from pregnant animals. Under flow, virgin and pregnant RAs constricted at predicted *in vivo* flow rates (11.8µl/min virgin vs. 60.4µl/min pregnant). Estrogen, progesterone, and PlGF (not VEGF), and the combination of all factors, delayed flow-mediated constriction in virgin RAs (p=0.04), enabling them to recapitulate the prolonged resistance to vasoconstriction with increasing flow rates seen in pregnancy. L-NAME inhibition of eNOS partly reduced RA reaction induced by individual paracrine factors, but this was insufficient to result in an effect when all paracrine factors were combined (p=0.95).

Conclusions: Maladaptation of RAs could cause over-sensitivity of vessels to flow, thus limiting utero-placental flow. While nitric oxide seems to be involved in the adaptation of vascular reactivity, additional mechanisms are yet to be uncovered. This will identify targets to improve vascular adaptation in the future.

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Expression of transcription factor NR4A2 is not altered in placentas from cases of growth restriction or preeclampsia, but is reduced in hypoxic cytotrophoblast

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BACKGROUND: Nuclear Receptor Subfamily 4 Group A Member 2 *(NR4A2;* transcription factor) transcripts are elevated in the circulation of individuals whose pregnancies are complicated by preterm fetal growth restriction (FGR) and preeclampsia. In this study, we aimed to establish whether the increased transcripts originated from the dysfunctional placenta, and to uncover the function of placental *NR4A2.*

METHODS: *NR4A2* mRNA expression was assessed in preterm and term placentas (qPCR). *NR4A2* expression and protein was assessed in placenta from cases of preterm preeclampsia, FGR and preterm controls (qPCR and western blot). *NR4A2* expression and protein was assessed in term placental explant tissue and primary cytotrophoblast exposed to hypoxia (1% O_2 compared to 8% O_2). Small interfering RNAs were used to silence *NR4A2* in term primary cytotrophoblasts; expression of angiogenic, growth, apoptosis, inflammatory and oxidative stress genes were assessed. *NR4A2* expression was assessed in a model of tumour necrosis factor (TNF)-α-induced endothelial dysfunction using primary human umbilical vein endothelial cells, and treatment of 200μM pravastatin.

RESULTS: *NR4A2* expression was significantly higher in term compared to preterm placentas. *NR4A2* mRNA expression and protein were not altered in placentas from preterm FGR or preeclamptic pregnancies. Hypoxia significantly reduced cytotrophoblast, but not placental explant *NR4A2* expression. Silencing cytotrophoblast *NR4A2* expression under hypoxia did not alter angiogenic Placental Growth Factor, nor anti-angiogenic *sFlt-1* expression or protein secretion, but increased expression of cellular antioxidant, oxidative stress, inflammatory, and growth genes. Endothelial *NR4A2* expression was not altered with TNF-α-induced dysfunction, or pravastatin treatment.

CONCLUSION: The origin of increased circulating *NR4A2* transcripts in women with FGR and preeclampsia remains unknown. However, *NR4A2* is downregulated in cytotrophoblast under hypoxic conditions, and its expression regulates several important cellular pathways. Further studies are required to identify whether these changes in placental *NR4A2* are a protective mechanism, or a driver of placental dysfunction.

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The regulatory role of human endometrial organoid secretome on trophoblast migration and invasion

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Background: Successful placentation requires delicate communication between endometrium and trophoblast. The invasion and integration of trophoblasts into the endometrium during early pregnancy is crucial to placentation. Dysregulation of these functions is associated with various pregnancy complications, e.g. miscarriage and preeclampsia. The endometrial microenvironment exerts important influence on trophoblast cell functions. The precise effect and mechanism of decidual glandular epithelial cells on trophoblast functions remain uncertain. **Hypothesis:** We hypothesized that the secretome of human endometrial gland is involved in regulating trophoblast migration and invasion. **Methods:**Human endometrial tissues were obtained from endometrial biopsies with written consent. The endometrial organoids were established in matrix gel with a defined culture condition. They were treated with hormones mimicking the environment of the proliferative phase (Estrogen, E2), the secretory phase (E2+ Progesterone, P4) and early pregnancy (E2+P4+Human Chorionic Gonadotropin, hCG). RNA-seq and miRNA-seq were performed on the treated organoid to compare their transcriptomes. Organoid secretions were also collected for mass spectrometric analysis. The viability and invasion/migration of the trophoblasts were determined by XTT assay and transwell assay respectively. **Results:** Endometrial organoid model with high expressions of endometrial gland markers were successfully established. Spent medium from E2+P4+hCG-treated endometrial organoids, which mimics the pregnant decidual microenvironment, significantly enhanced the invasion and migration of the trophoblast when compared to those obtained from proliferative/secretary phases. All the treatments have no effect on trophoblast viability. Our results further demonstrated that hormone treatment up-regulated the S100A9 protein production by the endometrial gland via mir-3194 suppression, and thereby promoted trophoblast invasion and migration. **Conclusion:** By using a human endometrial organoid model, we demonstrated that hormonal stimulation of endometrial gland secretome during pregnancy may be crucial to regulate the functions of human trophoblasts.

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Endometrial uterine natural killer cells (uNK) cell numbers do not predict implantation success in an IVF population

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ABSTRACT: Endometrial uNK cells are proposed to have roles in endometrial angiogenesis, embryo implantation, spiral artery remodelling, trophoblast invasion, placental formation and pregnancy success. While there is no clear definition of what 'normal' endometrial uNK cell numbers are and a clear lack of conclusive evidence, uNK cell numbers are often used as a prognostic criterion for immunosuppressive intervention in women undergoing IVF. This study was designed to quantify uNK cells and determine their spatial distribution in relation to endometrial arterioles in patients with RIF and implantation success (IS).

METHODS: Endometrial pipelle biopsies were collected 6-8 days post-LH surge in natural cycles from women with RIF (n=14), women with implantation success (IS) (n=11) and women with potential RIF (PRIF) at the time of the study (n=9) from 2013 to 2015. uNK cell numbers and spatial distribution were investigated by standard immunohistochemistry protocols and quantified by deconvolution for single cell quantification using a Gaussian Blur and Yen algorithm.

RESULTS: uNK cell numbers and distribution was not significantly different in women with RIF compared to women with IS. uNK cell density was higher in regions distal to arterioles in both patient groups and this density was reduced with increasing POD. Furthermore, a significant reduction in uNK cell density was observed in women who had a previous pregnancy compared to those who had not, regardless of their current implantation status.

CONCLUSIONS: Our results demonstrated uNK cell numbers and their spatial distribution relative to endometrial arterioles were not altered in women with RIF compared to IS. Our data also demonstrated uNK cell density was significantly influenced by POD and prior pregnancy. Combined, these findings do not support the clinical value of using uNK cell numbers as a prognostic indicators of implantation success due to significant hormonal and immune influences such as POD and pregnancy.

1. J F Donoghue et al., Endometrial uNK cell counts do not predict successful implantation in an IVF population, Human Reproduction, Volume 34, Issue 12, December 2019, Pages 2456–2466, https://doi.org/10.1093/humrep/dez194

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Small extracellular vesicles as biomarkers of endometriosis

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BACKGROUND

Endometriosis is a debilitating disease affecting 10% of women of reproductive age characterised by the ectopic growth of endometrial-like lesions, often in the peritoneal cavity. Diagnostic delays are common due to the requirement of invasive laparoscopy and no clinically useful biomarkers exist. Small extracellular vesicles (sEV), secreted by cells as intercellular messengers, carry unique cargo signatures, have pathogenic roles (e.g., in metastasis) and could have biomarker potential.

AIM

To identify differentially expressed cargo proteins in sEV from peritoneal fluid (PF) and peripheral blood (PB) of endometriosis patients compared to non-endometriosis patients and determine their correlation within these fluids.

METHODS

We collected matched PF and PB samples from patients undergoing laparoscopy and grouped them based on endometriosis presence or absence. sEV were isolated by differential ultracentrifugation and validated for universal sEV markers using flow cytometry and Western Blotting. Particle morphology was observed with Transmission Electron Microscopy (TEM) and the concentration and size distribution measured by nanoparticle tracking assay (NTA). Quantitative Tandem Mass Tag (TMT) LC-MS/MS will be used to characterise sEV proteomes and determine differentially expressed proteins between groups and fluids.

RESULTS

N=13 PF and N=12 (paired) blood samples have been collected from N=6 controls and N=7 endometriosis patients (mean age 37 and 32 years, respectively). Validation TEM analyses showed vesicles with a cup-shaped morphology typical of sEV. The mode particle diameters were 120.5-211.7 nm for PF preparations and 107.3-151.7 nm for PB, within the expected range, with a mean particle concentration of 1.06×10^{13} particles/mL for PF and 2.29×10^{11} particles/mL for blood, respectively. Preparations were positive for Syntenin-1, TSG101, CD9, CD63 and CD81, indicative of sEV. Proteomic analysis is ongoing.

CONCLUSION

sEV have been successfully isolated from PF and blood. We expect to find endometriosis-specific sEV which could serve as biomarkers of endometriosis.

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The fate of SUSD2+ endometrial mesenchymal stem cells during decidualization

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Regeneration of the endometrial stromal compartment in premenopausal women is likely maintained by the perivascular endometrial mesenchymal stem/stromal cells (eMSC) expressing sushi domain containing 2 (SUSD2). The fate of SUSD2 eMSC during pregnancy and their role in decidualization is not fully known. The aim of our study was to determine the effect of progesterone on the stemness of the SUSD2+ eMSC isolated from non-pregnant uterine samples. Secondary objectives were to characterize the functional capacity including differentiation into the mesenchymal cell lineage and CFU assays of SUSD2+ eMSC isolated from decidua at full term and compare it to the capacity of those isolated from non-pregnant uterine samples. Progesterone treatment induced changes in the decidual gene expression profile in non-pregnant SUSD2+ eMSC. However, the major MSC membrane surface markers remained unchanged. Histological analysis revealed a significantly lower abundance of SUSD2+ eMSC in 1st trimester and full term samples compared to non-pregnant samples, p=0.0296 and 0.005, respectively. The differentiation and the colony forming capacity did not differ significantly between the cells isolated from non-pregnant and pregnant uterine samples. In summary, pregnancy reduced the abundance of SUSD2+ eMSC, however, eMSCs function remained intact. Our results suggest that SUSD2+ eMSC undergo decidualization proces in vitro, while maintaining MSC membrane surface phenotype. Therefore, eMSCs likely play an important role in the course of endometrial decidualization and embryo implantation.

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A high lactate low pH microenvironment created by the blastocyst promotes endometrial receptivity and implantation.

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Successful embryo implantation requires a synchronized dialogue between the receptive endometrium and blastocyst via locally produced soluble mediators. At the time of implantation, the blastocyst produces significant amounts of lactate, creating a microenvironment around the embryo characterized by high lactate (LA) and low pH. Whilst historically considered a 'byproduct' of metabolism, identification of a lactate specific receptor, GPR81, and recent work in cancer cells has established LA as an important signalling molecule, with roles in angiogenesis, ECM breakdown and immunosuppression, processes that are vital for successful implantation. Hence, this study investigated the role of LA as an early embryonic signal to facilitate endometrial remodelling for receptivity and implantation.

Functional changes to the endometrium were assessed by exposure of hormonally primed ECC-1 and Ishikawa cells to LA, LA + neutralized pH (nLA) or acidic pH (pH_L), before analysis of tight junction integrity (TER), proliferation and gene expression changes. LA's effects on endometrial stromal cell decidualization, migration, as well as HUVEC endothelial tube formation/angiogenesis, were also determined.

TER and proliferation were downregulated in ECC-1 cells exposed to 2.5, 5 and 7.5mM LA (P<0.01), while exposure to nLA or pH_L alone had no significant effect. Upregulation of GLUT4, GPR81, VEGF, SNAI1, and RELA mRNA expression was observed following exposure of Ishikawa cells to combined LA + pH_L (P<0.05), while MCT-1 expression decreased. Additionally, LA increased the migration of decidualized stromal cells (P<0.05) without changing the extent of decidualization. HUVEC tube formation was significantly increased by 5mM LA exposure (P<0.01).

Together, these results suggest that the specialized microenvironment created by the blastocyst, of both high lactate and low pH, enhances endometrial remodelling for receptivity and implantation through functional changes to epithelial, stromal and endothelial cells in the endometrium. Therefore, LA appears to be an important signalling molecule in the maternal-fetal dialogue underpinning implantation.

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Proteomes of endometrial stromal cell-derived small EVs indicate decidualisation potential

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Background: Proper decidualisation is vital in preparation for endometrial health including, embryo receptivity, controlled cytotrophoblast invasion and subsequent endometrial regeneration. **Aim**: To investigate the role of small extracellular vesicle (sEV) mediated communication during decidualisation.

Methods: Human endometrial stromal cells (SC) were treated with estrogen and progesterone for 14 days (d) and grouped as well (WD) and poorly decidualised (PD) based on secreted prolactin level. sEV were isolated from conditioned media using differential centrifugation and subjected to mass spectrometry-based quantitative proteomic analysis.

Results and conclusions: On d2 PD- versus WD-SC-sEVs; 58 proteins were differentially regulated (DE) with 17 down-regulated (involved in complement/coagulation cascades, platelet degranulation and fibrinolysis) and 39 up-regulated (involved in focal adhesion, glycolysis /gluconeogenesis, PI3K-Akt signaling pathway and leukocyte transendothelial migration). On d14, in PD- versus WD-SC-sEVs, FLNA was down-regulated while 21 proteins were up-regulated associated with complement/coagulation cascades (C3, C6), platelet degranulation (SERPINA4, ITIH4), B-cell receptor signaling and innate immune response (immunoglobulins). Six proteins, PRDX1, PFN1 VCL, RAC1, THBS4 and ANXA1 (for invasion, migration and embryo implantation) were uniquely present in WD-sEVs. Interrogation of changes from d2 to d14 identified no significant changes in WD-SC-sEVs, however, in PD-SC-sEVs, proteins involved in complement and coagulation cascade were significantly upregulated at d14 while 36 proteins (involved in focal adhesion, regulation of actin cytoskeleton and glycolysis/gluconeogenesis were only present in d2.

Our findings provide an insight into sEVs as a mode of cellular communication by SC, provides insight into sEVs-proteomes as a benchmark of well decidualised SC and how failure of appropriate decidualisation may lead to dysregulated communication that is critical for embryo implantation, enabling and limiting trophoblast invasion during placentation and sensing a healthy embryo.

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Is exocytosis the key to detecting uterine receptivity: examination of extracellular vesicles, porosomes and SNARE proteins

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Luminal uterine epithelia cells (UECs) have a surge in vesicular activity during early uterine receptivity. Recent studies have highlighted the importance of uterine extracellular vesicles (EVs) in maternal-foetal communication in a number of species including humans. These vesicles are thought to exit the UECs via exocytosis transport mechanisms. Exocytosis mechanisms in UECs during uterine receptivity were investigated with transmission electron microscopy (TEM) and via localisation and quantification of various SNARE proteins involved in exocytosis (VAMP2, syntaxin 2, 3 and SNAP23). All SNARE proteins investigated were found in the apical cytoplasm of UECs at the time of receptivity and exhibited significantly higher abundance at this time compared to the time of fertilisation. Interestingly, SNAP23 was identified in the luminal fluid exclusively at the time of uterine receptivity. TEM examination further localised a variety of EVs in the uterine luminal space at the time of receptivity. Porosomes, a plasma membrane ultrastructure involved in “kiss and run” exocytosis, were found to be significantly increased at receptivity using quantitative TEM analysis. Overall, this study found at an ultrastructural level there is an increase in vesicular activity via EVs and porosomes at the time of receptivity. Our morphological and protein results suggest that exocytosis occurs predominantly at the time of receptivity in the rat utilising a variety of different exocytosis routes. This work has also shown that this increase in exocytosis is controlled by SNARE proteins, which are therefore responsible for creating the utmost microenvironment for blastocyst implantation. In particular, the presence of SNAP23 in the luminal fluid exclusively at the time of receptivity has immense potential for use as a receptivity marker, which could be used to improve artificial reproductive technologies and our understanding of uterine receptivity during early pregnancy.

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The regulatory roles of human endometrial gland secretions at the fetal-maternal interface

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The success of pregnancy depends on a well-established fetal-maternal interface, which consisting of endometrial stromal/epithelial cells, immune cells, endothelial cells and trophoblasts. The endometrial gland, which is composed of glandular epithelial cells, is essential for the survival/development of conceptus by secreting and transporting various paracrine factors. We hypothesized that the soluble factors derived from endometrial gland regulate the decidual macrophage polarization and stromal cell functions. A long-term human endometrial organoid culturing system was established by using endometrial glandular tissue. The derived organoids were treated by sex hormones estrogen (E2), progesterone (P4), and/or human chorionic gonadotropin (hCG) to mimic the estrous cycle and early pregnancy environment. The endometrial organoids can be maintained for long-term and functionally respond to E2 and P4. When further stimulated with pregnancy hCG, they acquired characteristics of gestational endometrium as demonstrated by increased glycodelin-A production. Human monocytes were isolated from female blood by immunomagnetic separation and were differentiated into macrophages using macrophage colony-stimulating factor (M-CSF; 50 ng/ml). The inclusion of the E2+P4-treated endometrial organoid secretome during the M-CSF-induced differentiation increased the expression of decidual macrophage marker indoleamine 2, 3-dioxygenase 1 (IDO-1) in the resulting macrophages. The phagocytosis of the differentiated macrophages was inhibited by the secretome of E2+P4+hCG-treated endometrial organoids. An endometrial organoid and stromal cell co-culture model was also established to study the effect of paracrine factors of endometrial glands on decidualization. Our results showed that endometrial organoid secretome suppressed the progestin-cAMP-induced decidualization of the stromal cells. In conclusion, hormonal treatments modulated the secretome of organoids and thereby their actions on endometrial cells.

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Attachment-requisite E-cadherin expressed on apical surface of endometrial epithelial cells is regulated by p120-catenin

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Understanding orchestration of human embryo and endometrium during early implantation helps to improve the outcome of pregnancy. At the beginning of implantation, competent embryos can modify endometrium through secreted paracrine signals. Apical surface of endometrial epithelial cells (EECs) is the first part on the maternal side interacting with embryo during implantation. Using a human embryonic stem cells derived trophoblastic spheroids (BAP-EB), we previously showed that antibody blocking of E-cadherin on the surface of receptive Ishikawa cell line significantly reduced BAP-EB attachment rates. It was reported that p120-catenin is essential for E-cadherin stability. In this study, we further examined the roles of p120-catenin and E-cadherin during early implantation process. Using live-cell immunofluorescent staining, we confirmed the expression of E-cadherin on the apical surface of Ishikawa cell line and primary EEC isolated from human endometrial aspirates at 7/8 days after luteinizing hormone surge (LH+7/8). We further found that conditioned media (CM) collected from attachment competent BAP-EB differentiated for 72h significantly induced the expressions of membranous bound p120-catenin when compared to CM collected from attachment incompetent BAP-EB differentiated for 48h. Furthermore, knockdown of p120-catenin in Ishikawa cells by siRNA transfection significantly reduced E-cadherin expression in Ishikawa cells. Concordantly, p120-catenin knockdown also significantly reduced the BAP-EB attachment rates and spreading areas. Most importantly, immunoblotting results of primary EEC collected at LH+7/8 day showed that the protein levels of E-cadherin and p120-catenin were significantly higher in EEC collected from women who ultimately gave livebirth (n=27) when compared to those collected from women who were not pregnant (n=35). Our results suggest that the competent embryo might induce the expressions of endometrial p120-catenin which maintain the apical expression of E-cadherin during implantation process. The current data further prompt to the use of p120-catenin and E-cadherin as potential receptivity markers for predicting pregnancy outcomes.

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The role of prorenin and the (pro)renin receptor in successful placentation

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The renin-angiotensin system (RAS) has roles in placentation, by regulating trophoblast invasion, uterine spiral artery remodelling, and angiogenesis. We have shown that the (pro)renin receptor ((P)RR), which can activate the RAS cascade, promotes proliferation, migration, and invasion of trophoblast cells *in vitro*. This study aims to assess the physiological role of (P)RR in placentation *in vivo*.

GFP-expressing lentiviral packaged gene-constructs were used to specifically knock-down (P)RR expression in the trophectoderm of mouse blastocysts (Chakraborty et.al., *Bio-protocol*, 2017). Zygotes were collected from super-ovulated C57/BL6/CBA F1 female mice and cultured until they reached the blastocyst stage. Blastocysts were then exposed to Acid-Tyrode's solution for 30s and washed in G2 media, removing the zona pellucida. Blastocysts were incubated for 6h in either vehicle (no virus), control lentivirus (containing vehicle shRNA and GFP; $1x10^8$VP/ml), or (P)RR knockdown virus (containing (P)RR shRNA and GFP; $1x10^8$VP/ml) before being cultured in modified-RPMI media for 96h to examine GFP and (P)RR expression in blastocyst outgrowths. In separate experiments, blastocysts were transferred into recipient pseudo-pregnant Swiss female mice, after which fetal and placental tissues will be collected at embryonic day-10 and 18.

Treatment with both (P)RR knockdown or control lentivirus [$1x10^8$VP/ml] increased GFP expression in blastocysts 96h post-transfection, compared to the vehicle control. No significant GFP expression was observed at earlier timepoints. Treatment at $0.5x10^8$VP/ml, compared to the $1x10^8$VP/ml treatment group exhibited sparse, less uniform GFP expression. Initial assessment of embryos infected with control or knockdown virus and transferred into female recipients are underway. We will collect maternal and fetal tissue to assess trophoblast-specific GFP expression, maternal blood to measure plasma s(P)RR levels and placentae to determine the role of (P)RR in placentation.

Given that (P)RR is involved in various trophoblast cell function, we expect that knocking down placental (P)RR will impair placental development and reduce fetal growth.

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Regulation of human endometrial receptivity by microRNAs for embryo implantation

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Background: Embryo implantation is a key step in establishing pregnancy and a major limiting factor in IVF. The inner lining of the uterus, the endometrium, must transform from non-receptive to receptive to allow embryos to implant but the mechanisms governing endometrial receptivity are not well understood. Our laboratory recently discovered that glycoprotein podocalyxin (PODXL) is an important negative regulator of human endometrial receptivity. PODXL is highly expressed on the apical surface

of all epithelial and endothelial cells in the non-receptive endometrium, but down-regulated specifically in the luminal epithelium at receptivity. Furthermore, PODXL inhibits embryo attachment and invasion, demonstrating that down-regulation of PODXL is essential for implantation. Our previous study indicated that this down-regulation is mediated by progesterone, however, the detailed molecular regulations are unknown. **Aim**: To investigate the role of microRNAs (miRNAs) in regulating PODXL for receptivity. **Methods**: Primary human endometrial epithelial cells were isolated from women, cultured in the presence of estrogen (E, mimics the non-receptive state) or estrogen plus progesterone (EP, mimics the receptive state), and screened against 13 bioinformatically predicted miRNAs that may target PODXL. The miRNAs found to be significantly regulated by hormonal treatment were subsequently validated by real time RT-PCR, and their functional importance was determined in *in vitro* embryo implantation models. **Results**: Two miRNAs were found to be significantly up-regulated in EP compared to E treated cells. To confirm the functional importance, these miRNAs were transfected into endometrial cell line Ishikawa cells in the absence of progesterone, and both significantly down-regulated PODXL at both the mRNA and protein levels. Moreover, both miRNAs significantly enhanced embryo implantation in *in vitro* models. **Conclusions**: This study identified specific miRNAs that are up-regulated by progesterone which target PODXL to promote endometrial receptivity; these results may potentially lead to therapeutics to improve IVF success in those with endometrial-related infertility.

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Culture and differentiation of term side-population trophoblasts

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Objective: The ability to isolate human trophoblast stem cells (TSC) from third-trimester placentae is key to understanding major pregnancy pathologies, but has proved challenging to date. We have previously used the side-population technique to isolate TSC-like cells from first trimester placentae that have gene expression characteristics and the differentiation potential of a TSC population. The same side-population technique can also isolate these cells from third-trimester placentae, but it is apparent that different trophic requirements are required to sustain cell viability at this gestation. This work aimed to optimise extracellular matrices and cytokine supplementation to improve third-trimester side-population trophoblast viability and differentiation.

Methods: The Hoechst side-population technique was used to isolate trophoblasts from normal third-trimester placentae. The effect of different extracellular matrices (5µg/mL Collagen-IV, or 10µg/mL Laminin-521), or the addition of 25ng/mL Decorin and/or 50ng/mL IL-8 on cell adherence, viability, and growth over 14 days of culture was assessed using ilastik and CellProfiler software.

Results: Twice as many third-trimester side-population trophoblasts attached and spread across the culture surface on Laminin-521 (41±2.000 SEM, n=3) than on Collagen-IV (17.33±6.333 SEM, n=3, p=0.1000), and most cells were entirely lost from the Collagen-IV surface by day 14 in culture. Compared to controls (0.008 mm^2±0.0007 SEM, n=3), addition of either decorin (0.188mm^2±0.0038 SEM, n=3, p<.0001) or IL-8 (0.241mm^2±0.0043 SEM, n=3, p<.0001) significantly increased the size of side-population colonies over 14 days. Combining decorin and IL-8 supplementation enabled third-trimester side-population trophoblasts to be maintained for at least 30 days of culture (n=3). Preliminary data suggests side-population cells can differentiate into syncytin-1 positive syncytiotrophoblast (n=2) or HLA-G positive extravillous trophoblast (n=1).

Conclusion: The optimisation of conditions to allow 2D culture and manipulation of third-trimester side-population trophoblasts in an undifferentiated state underlie further analyses of potential trophoblast dysfunction in pregnancy pathologies.

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The first transcriptomic profile of equine endometrial glands provides a novel insight into endometrial gene expression

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Early pregnancy loss has major economic implications for horse breeders, and despite there being many studies focusing on conception and embryo loss, the mechanisms by which the uterus is primed for pregnancy are yet to be elucidated in this species. To advance knowledge in this space, the present study aimed to characterise the transcriptome of epithelial glands derived from the equine endometrium. Two endometrial tissue samples were collected from two mares immediately post-mortem, and epithelial gland cells were isolated via enzymatic digestion. Contaminating stromal cells were removed by selective adhesion. RNA was isolated from purified gland cells before being sequenced using DNB-Seq technology. Epithelial gland cell sequencing data were analysed at SAHMRI and bioinformatics analysis was completed using protein analysis through evolutionary relationships (PANTHER). A total of 13,038 unique genes were identified by RNA-Seq with >2 counts per million (CPM) in each sample. Amongst the most abundant protein-coding genes were MT-CO1, EEF1A1, EGR1, HSP90AA1 and CD74, with roles in cellular respiration, translation, transcription regulation and the innate immune system, respectively. PANTHER analysis revealed 157 active pathways, the most prominent being Wnt signalling, chemokine and cytokine mediated inflammation, GNRH receptivity, integrin signalling and angiogenesis. Additionally, biological functions associated with the immune system and reproduction comprised 2.3% and 0.6% of the identified genes respectively. In conclusion, this study is the first to characterise the transcriptome of equine epithelial gland cells and provides novel insights into the functions of genes expressed in this cell type.

These findings establish a foundation for the development of novel *in vitro* models, such as a 3-dimensional endometrial organoid model, required for the advancement of knowledge surrounding uterine priming and conception in the mare.

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Characterizing endometrial stem/progenitor cells in menstrual fluid in women with and without endometriosis

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Endometrial stem/progenitor cells identified in menstrual fluid (MF) and endometrial tissue are proposed to cause endometriosis.

We aimed to compare endometrial stem/progenitor cells in MF from women with and without endometriosis.

MF (day 2) from a menstrual cup (case n=5, control n=4; mean±SD: 34.7±9.6yrs) was collected. MF was dissociated to single cells, and leukocytes depleted using CD45 magnetic beads. Stem/progenitor cell proportions were determined by flow cytometry (%N-cadherin$^+$ or SSEA1$^+$ of EpCAM^{Hi}CD45$^-$CD31$^-$ epithelial cells or %SUSD2$^+$ from CD45$^-$CD31$^-$ endometrial cells) and clonogenicity by colony forming assay.

There was no significant difference between epithelial (EpCAM$^+$CD45$^-$CD31$^-$) cells in MF (case: 26.0±6.9%; control: 31.8±9.4%, p=0.29. Epithelial progenitor cells (SSEA1$^+$ and/or NCAD$^+$) and mesenchymal stem cells (SUSD2$^+$) cells were found in all samples. There was no significant difference in epithelial progenitor cell populations in MF, for SSEA1$^+$ (case: 2.5±3.2%; control: 2.2±2.0%, p=0.56), NCAD$^+$ (case: 11.4±11.5%; control: 6.6±4.7%, p=0.90) or NCAD$^+$SSEA1$^+$ (case: 0.6±0.7%; control: 1.5±2.2%, p=0.40). There was no difference between %SUSD2$^+$ in case and control MF (case: 11.2±7.4%; control: 9.9±5.0%, p>0.99). We noted a higher proportion of total viable CD45$^-$CD31$^-$ endometrial cells in endometriosis MF compared to control MF after beading (case: 68.9±14.3%; control: 14.5±5.8%, p=0.02). Epithelial and stromal clonal efficiency appeared similar between control and endometriosis MF.

MF contains endometrial stem/progenitor cells in proportions that may reflect eutopic endometrium. An increased sample size/power may reveal differences of disease modelling potential.

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An exploratory study for using extracellular vesicle miRNA as a biomarker of fertility status

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Decline of fertility links to genetic traits. Identification of novel biomarkers has resulted in improvements of fertility treatments. Exosomes (EX) are small extracellular vesicles (EVs) of ~30-150nm diameter which have gained recognition as a prognostic and diagnostic biomarker of several disorders. Exosomes enhance inflammatory mediators in the endometrium and uterus, which can lead to infertility. EX contain, among other biomolecules, micro-RNA (miRNA) which have been validated as biomarkers in various diseases including cancer. MiRNAs have been shown to regulate post-transcriptional modifications and are differentially expressed between divergent groups of fertility.

The present exploratory study aims to evaluate the miRNA profiles of EV and fractionated exosomal samples of high and low tick-resistant beef cattle and their offspring, to explore the potential of miRNA biomarkers of tick resistance. A novel tick scoring system was adopted to classify cows (n = 3/group) into high or low tick resistant groups. Established isolation and enrichment protocols were used to isolate EVs and fractionate EX from the bovine blood plasma. The resultant EX and non-EX samples were processed for next generation miRNA sequencing.

MiR-449a, relates to cellular inflammatory pathways, was highly expressed in maternal high tick-resistant EX samples, in which a total of 2631 miRNAs were identified in fractionated EX and non-EX samples. Of these, 174 novel miRNAs were identified, and 10 were differentially expressed (DE) (FDR < 0.05) (Figure 1). EV samples also contained these 10 DE miRNAs, and three miRNAs were highly expressed: miR-2419-3p, miR-7861-3p and miR-2372-5p. Enrichment analysis shows that these miRNAs alter cellular signalling pathways relate to inflammation. Fractionated samples of offspring contained 196 novel miRNAs, however no miRNA were differentially expressed.

The findings of this exploratory study demonstrate the potential of EV, EX and non-EX miRNA as biomarkers of genetic disorders which can be applied to fertility biomarker studies.

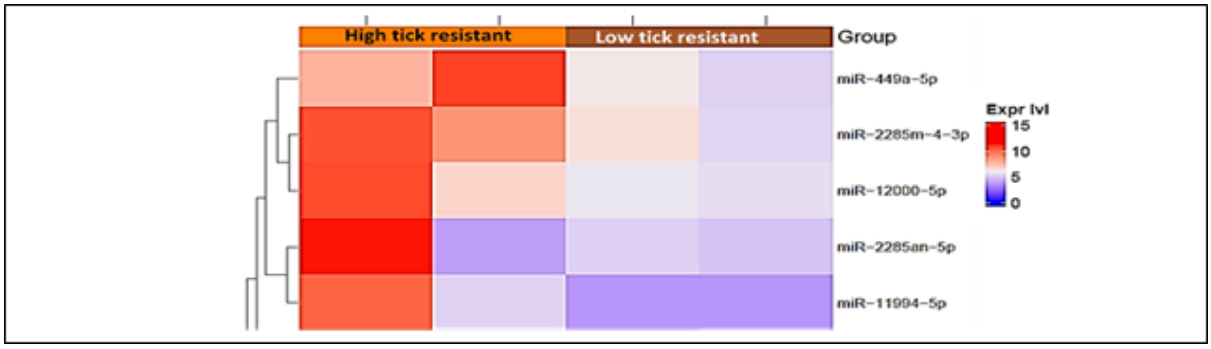

Figure 1. Heatmap of differential expression of miRNAs between high and low tick resistant mother plasma SEC samples (EX and non-EX)

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Amino acid supplementation affects fertilisation of bovine oocytes *in vitro*

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In vitro fertilisation (IVF) can be used to improve herd genetics by combining gametes from superior animals, resulting in significant commercial returns for the cattle industry. Adoption of IVF in breeding programs has been limited as pregnancy rates are lower compared to traditional technologies such as artificial insemination and embryo transfer. Improving IVF culture conditions to support the metabolic activity of oocytes and spermatozoa will increase the proportion of viable preimplantation embryos. It has been demonstrated previously that the combination of glutamine (Gln), proline (Pro) and Isoleucine (Ile) support bovine oocyte maturation, whilst Cystine (Cys2) caused a significant reduction in nuclear maturation rates. These amino acids (AAs) could also affect oocytes and/or spermatozoa during fertilisation. Therefore, the aim of this study was to investigate the effects of *in vitro* supplementation of Gln, Pro, Ile and Cys2 during fertilisation on the formation of 2 pronuclei (2PN).

Bovine oocytes matured in TCM199 were fertilised in FERT-TALP supplemented with either 1 mM Gln, 0.4 mM Pro, 0.28 mM Ile or 0.07 mM Cys2 for 20 h. The %2PN for oocytes supplemented with 1 mM Gln (56.5 ± 2.4), 0.4 mM Pro (57.7 ± 6.6), 0.28 mM Ile (60.3 ± 3.8) was not significantly different to those in FT alone (60.9 ± 2.0). However, the %2PN was significantly reduced with the addition of Cys2 (42.5 ± 1.1) compared to FT alone ($P \leq 0.05$). Our results suggest that the addition of Gln, Pro or Ile individually, during IVF does not increase the %2PN, though further investigation is required to determine whether supplementation with these AAs during fertilisation affects embryo development. Furthermore, Cys2, which is often used in multiple culture media formulations, caused a reduction in the proportion of zygotes following IVF, and these deleterious effects on fertilisation are yet to be determined.

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Cyclic changes in cortisol across the estrous cycle in parous and nulliparous Asian elephants

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Glucocorticoids (GCs) are generally considered to have negative effects on reproduction. However, in well-studied model species, GCs fluctuate predictability across the oestrous cycles, and short-term increases promote healthy ovarian function. Reproductive challenges have plagued captive elephant populations, which are not currently self-sustaining. Efforts to understand reproductive dysfunction in elephants have focused on the suppressive effects of cortisol, but the potential permissive or stimulatory effects of cortisol are unknown. In this study, we provide a detailed examination of cortisol patterns across the oestrous cycle in Asian elephants (Elephas maximus). Time series analysis was used to analyse cortisol and progesterone data for a total of 73 cycles from eight females. We also compared cortisol profiles between females that successfully conceived and females that failed to conceive despite repeated mating attempts. Our results revealed that cortisol fluctuates predictably across the oestrous cycle, with a peak during the second half of the follicular phase followed by low levels throughout the luteal phase. Furthermore, this pattern was significantly altered in nulliparous females; cortisol concentrations did not decline during the luteal phase to the same extent as in parous females. This study highlights the complexity of cortisol signalling and suggests future directions for understanding the role of cortisol in reproductive dysfunction.

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Novel endometrial epithelial organoids for modelling gynaecological disease

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Endometrial epithelial organoids (EMO) are an important tool for gynaecological research, but have been limited by generation from 1) invasively acquired tissues, often from advanced disease states and 2) from women who are not taking hormones, thus

excluding 50% of reproductive age(d) women. Here we sought to overcome these limitations by generating endometrial epithelial organoids from 1) menstrual fluid (MF; MFO) using a method enabling concurrent isolation of menstrual fluid supernatant, endometrial stromal cells and leukocytes and 2) from biopsies and hysterectomy samples from women taking hormonal medication (EMO-H). MF was collected in a menstrual cup for 4-6 hours on day 2 of menstruation, biopsies and hysterectomies were obtained during laparoscopic surgery. Organoids were generated from all sample types and their proliferation and cell surface markers were characterized. Flow cytometry markers included Epithelial cell adhesion molecule, Neural-cadherin and Stage-Specific Embryonic Antigen-1. MFO and EMO-H replicated EMO; all showed low levels of expression of the endometrial basalis epithelial cell marker SSEA-1 and had similar rates of cellular proliferation. Our results demonstrate that MFO and EMO-H are novel organoids that replicate standard EMO with the advantage of being derived 1) non-invasively, whilst also enabling concurrent isolation of other menstrual fluid components and 2) from 50% of the population that currently are not being studied with standard methods. Thus, MFO and EMO-H are likely to prove invaluable tools for gynaecological research, enabling population wide assessment of endometrial health including from adolescents.

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You are what your mother eats: maternal macronutrient intake and the effects on offspring metabolism

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It is well accepted that *in utero* exposure to maternal diet can program offspring body composition and susceptibility to disease in later life. While animal studies have focused primarily on the effects of either maternal under-nutrition (e.g., calorie or protein restriction), or over-feeding of high fat diets, little is known about the effects of maternal macronutrient balance (i.e., the proportions of protein, fat, and carbohydrate in the diet) in modulating offspring health. An important model called *protein leverage* explains that in many animals, including humans, protein is prioritised over carbohydrates and fats when confined to imbalanced diets. This tight, innate regulation of protein intake, influenced by dynamic protein targets, can result in the overconsumption of fats and carbohydrates when given protein-poor diets. However, the question remains as to *when* and *how* this strong regulation is programmed in an individual. We hypothesise that protein targets may be determined i*n utero* and through early life programming. Using a mouse model, we investigate how maternal protein to carbohydrate (P:C) balance influences offspring protein-specific appetite and metabolic health. We show that offspring from dams fed high P:C diets throughout gestation and lactation have greater protein targets and increased body weights in early life, a result consistent across sexes. We also show that these greater protein targets increase offspring food intake when placed on no-choice diets, resulting in an overall increase in body weight and fat mass. The combination of a high protein maternal diet and a Western diet in adulthood is revealed to further exacerbate this obese phenotype. This work highlights the massive implications of early life programming on later life metabolism. It could aid in explaining known patterns in the epidemiology of obesity and will provide fundamental new understanding of the ways in which maternal nutrition shapes offspring health.

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Small-molecule factors improve development of mouse preimplantation embryos by protecting them against oxidative stress

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Culture of mouse zygotes at low density to the hatching blastocyst stage in medium containing selected small molecules improved development from the 5-cell stage through to the hatching blastocyst stage. Here, we show that these factors reduce mitochondrial membrane potential, reduce reactive oxygen species (ROS) levels, and increase glutathione (GSH) levels. Mitochondrial membrane potential and reactive oxygen species (ROS) levels in 2-cell and 4-cell stage embryos that had been cultured from the zygote stage in medium containing small-molecule factors were examined using tetramethylrhodamine methyl ester (TMRM) and 2',7'-dichlorofluorescein diacetate (DCFDA), respectively. The small-molecule factors reduced mitochondrial activity at both the 2- and 4-cell stages by ≥40% and reduced ROS by >60%. Similarly, staining of 2-cell and 4-cell embryos for GSH levels with tetrafluoroterephthalonitrile indicated that culture of zygotes in the presence of small-molecule factors increased GSH levels. This was supported by data showing an increased GSH/GSSG ratio throughout all stages of preimplantation development, as determined by mass spectrometry. The results are consistent with the 'quiet embryo' hypothesis that reduced metabolic activity and ROS favours developmental progression. In summary, these results indicate that small-molecule factors reduce ROS by a combination of suppressing the activity of mitochondria (a major source of cellular ROS) and increasing the concentration of the antioxidant GSH. These results provide insight into how specific factors can be used successfully to improve embryo development *in vitro* by reducing oxidative stress.

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Expression of podocalyxin in high grade serous carcinoma and its potential role in facilitating spheroid formation

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Background: High grade serous carcinoma (HGSC) represents one of the most aggressive forms of ovarian cancer, accounting for the majority of advanced-staged cases. HGSC often spreads transperitoneally in cell clusters known as spheroids, which detach from the primary tumour and enter the fluid of the abdominal cavity. Overexpression of glycoprotein podocalyxin (PODXL) has been associated with a significant decrease in disease-free survival in HGSC patients (1). This study aimed to examine PODXL expression in HGSC tissues and ovarian cancer cell lines, and to investigate whether PODXL expression is associated with cancer spheroid formation.

Methods: PODXL protein was examined by immunohistochemistry on a tissue array containing 80 patients with epithelial ovarian cancer. Over 37 ovarian cancer cell lines were assessed bioinformatically and the results were confirmed by real-time RT-PCR analysis in SKOV3, Kuramochi, HEY and COV362 lines as representatives. These four lines were then cultured on ultra-low attachment plates to form spheroids and PODXL localisation was determined by immunocytochemistry and confocal imaging.

Results: The tissue array showed positive PODXL staining in 85% of HGSC cases, with 30% being at advanced stages (FIGO stage III and IV). Bioinformatic analysis revealed wide PODXL expression in ovarian cancer cell lines with the highest level detected in Kuramochi, a model HGSC cell line. These data were validated by real-time RT-PCR analysis of four representative lines, confirming Kuramochi expressing the highest level followed by HEY, SKOV3 and COV362. The ability of spheroid formation also followed this order, with COV362 which expressed low levels of PODXL failing to form compact spheroids. PODXL was localised to the surface of the spheroids.

Conclusions: PODXL is widely expressed in HGSC tissues and ovarian cancer cell lines, and positively correlates to the ability of cancer spheroid formation. These data suggest an important role of PODXL in promoting cancer metastasis in HGSC.

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A maternal high-sugar diet promotes body adiposity in offspring

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Maternal diet quality is an important and easily modifiable factor linked to offspring health. In human and animal studies, maternal obesity and impaired fetal growth are predictors of poor cardiometabolic health later in life. Carbohydrates are the most abundant macronutrient in the diet and glucose is the primary energy source for a fetus, therefore, carbohydrates quality is paramount to good metabolic health. Glycaemic index (GI) is a measure of carbohydrate quality based on postprandial blood glucose levels. Low dietary GI is associated with weight gain prevention and better metabolic health. This study compared diets high in free sugars varying in GI (glucose (high), sucrose (moderate) and isomaltulose (low)). Sucrose and isomaltulose are both glucose-fructose disaccharides, however, bond positioning alters their digestion and blood glucose response. C57BL/6 female mice were fed one of these three sugar-based isocaloric diets or an AIN93G control and mated after 5-weeks. Half the dams were culled at day 18 of pregnancy and fetuses collected (n=10 litters/diet). The remaining dams gave live birth and their pups continued on their mother's diet until 30-weeks (n=10 litters/diet). EchoMRI determined body composition. At the end of the pre-pregnancy feeding stage all sugar dams had gained more weight than control dams (p<0.001 vs chow) and sucrose and isomaltulose dams were fatter (p<0.001 vs chow). During pregnancy all sugar dams were fatter than control dams (p<0.001 vs chow), however, only glucose dams gained more weight during pregnancy (p<0.05 vs chow). At day E18 of pregnancy, glucose pups (p<0.05 vs chow) and their placentas (p<0.001 vs chow) were heavier than fetuses from all other diets. By 30-weeks all sugar-fed female pups were heavier and fatter than AIN93G pups (p<0.01 vs chow). These results suggest that maternal carbohydrate type, regardless of GI and saccharide composition effect offspring body weight and composition in later life.

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Role for Selenium in homeostasis and reproduction

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Background: The essential micronutrient Selenium (Se) is critical for human health. The thyroid gland contains the highest Se concentration; seleno-proteins are responsible for thyroid hormone (TH) synthesis. Moderate Se-deficiency may impact seleno-protein function and impair TH synthesis leading to metabolic disorders and infertility. While Se supplementation improves clinical outcomes, there is no consensus on the correct dose and form of dietary Se for humans, leading to conflicting guidelines on optimal dietary Se concentrations.

Aims: This project provided male C57BL/6 mice standard chow (control, 0.3ppm/kg) and three dietary Se forms (chow-containing selenite (NaSe, 5ppm/kg), methylselenocysteine (Met 10ppm/kg) and diphenyl diselenide (DDS 15ppm/kg)) to assess their potential in thyroid/reproductive health, and body metabolism.

Methods and Results: EchoMRI studies revealed that the DDS diet decreased body weight significantly including lean and fat mass. However, circulating TH levels in the same mice was identical to the controls as determined by quantitative ELISA, which suggests that reduced lean/fat masses were likely due to lower food intake rather than enhanced metabolism. By contrast, mice fed with dietary NaSe and Met trended to lower fat mass, although lean mass/body weight were comparable to the control. These outcomes suggest enhanced body metabolism in mice receiving dietary NaSe and Met, as a corresponding elevation in TH and thyroid-stimulating hormone (TSH) was observed in these same mice. Interestingly, NaSe and Met diets caused significant

increase in sperm motility consistent with enhanced testicular function, while mice taking the DDS diet showed significantly lower testicular weight, sperm count and motility compared to NaSe, Met and control.

Conclusions: Taken together, our data indicate that dietary NaSe and Met improved thyroid/testicular function, enhanced body metabolism and weight loss with a concomitant enhancement of factors affecting male fertility, further studies are warranted to evaluate the levels, activities, and seleno-toxicity with reference to thyroid/testicular functions.

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Oestrogenic metabolite equol reduces reproductive capacity in *drosophila melanogaster* over two generations

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The role of phytoestrogens as endocrine disrupting agents is of emerging relevance in reproduction, as transgenerational impacts continue to be uncovered. To investigate whether oestrogen-like compound equol could impact reproduction over subsequent generations, a *drosophila melanogaster* model was used. Male and female flies (P1) were exposed from hatch to a period of peak fertility, to a physiologically relevant level of dietary equol (5mm). P1 were then either mated to a non-exposed partner of the same age, or an exposed partner. The subsequent offspring (F1) were then mated to either a partner from exposed or non-exposed parents. All matings were done in replicates of 10. The seminal vesicles of 5 males per group, per generation were also measured. In the P1 generation, exposure to equol reduced egg-producing capacity from both males and females regardless of whether they were mated to a control or exposed partner (control 31.25 ± 2.9 eggs per lay vs exposed female 20.27 ± 2.7 vs exposed male 15.08 ± 2.3, P=0.006). The number of subsequent offspring (F1) was also reduced (control 16 ±3.1 offspring per mating vs exposed P1 female 5.6 ± 0.3 vs exposed P1 male 2.5 ± 0.5, P<0.001). F1 flies with either a male or female equol-exposed parent had reduced egg-producing capacity (control 48.5 offspring per lay, vs 2.6 exposed female P1, 13.33 exposed male P1, P =0.015). Male F1 had reduced seminal vesicle size if either parent had been exposed to equol (control 0.0771 ± 0.002 mm^2, vs 0.057 ± 0.001 mm^2 from exposed female parent, 0.066 ± 0.001 mm^2 from exposed male parent, P =0.015). Male F2 had reduced seminal vesicle size if exposure occured on the maternal side (P= 0.006). Further work is warranted to determine how equol is able to program the reproductive capacity of exposed individuals and their descendants.

ANZBMS POSTER ABSTRACTS

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THE EFFECT OF LONG DISTANCE AIR TRANSPORT ON BONE MARKERS IN THOROUGHBRED RACEHORSES

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Long distance transport of horses to compete in races has increased in recent years. The effect of transport on behaviour and the immune system of horses has been studied however the effect of transport on the skeleton is less well understood. In the current study we investigated the effect of international transportation on bone turnover. International horses (IH; n=69) transported by air to participate in races in Melbourne and local horses trained in Melbourne (LH; n=79) were sampled. Two blood samples were obtained at 3-5 days post-arrival and 14 days later from IH, and at a 14-day interval from LH. Serum biomarkers - OCN and CTXI, cortisol, serum amyloid A, melatonin and bisphosphonates were measured. The relationships of the biomarkers and horse groups were determined using linear regression models. Entire males were over represented in the IH group, which also tended to be older than the local horses. IH had a higher cortisol than LH at the first sample (138.46 ± 162.14 nmol/L vs 84.81 ± 37.62 nmol/L; P<0.001) but were not different to LH after a further 14 days. IH had lower OCN and CTXI compared to LH but when adjusted for age and gender, only OCN was lower in IH than LH at timepoint 2 (15.85 ± 7.12 ng/mL vs 20.02 ± 7.98 ng/mL; P<0.001). The majority of measurable effects of international transportation in the current study had resolved at two weeks post transport. Differences in bone markers could mostly be explained by differences in age and gender between international and local horses, however post transport, IH show lower levels of bone formation than LH two weeks after arrival.

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Gene mining for novel molecular determinants of dental and skeletal homeostasis and disease in the Collaborative Cross

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The Collaborative Cross (CC) is an octo-parental recombinant inbred panel of mice, generated as a community resource by the CC Consortium to advance our knowledge and understanding of human disease. The Gene Mine (Geniad) is the WA breeding program of the CC, consisting of over 1000 strains, which have been housed by UWA since 2004. The aim of the CC is to provide a stable, reproducible genetic reference platform for the qualitative and quantitative analyses of causative gene-variants, epistatic mechanisms, and environmental factors which determine disease and characteristic phenotypes, including osteoporosis, cardiovascular disease, cancer, and diabetes. Integration of phenotypic and genomic data over time and across a variety of fields is vital to the delivery of the CC reference platform and advancing our understanding of disease susceptibility. Research of the phenotypic and genomic inter-relationships of the osteoporosis, osteoarthritis, and scoliosis fields of the CC has been initiated by our group. My research investigates the dental and skeletal fields of the Geniad CC population. Aims: 1) to identify novel molecular determinants of dental and skeletal homeostasis and disease, and 2) to integrate findings from the dental and skeletal fields across concomitant CC fields, including osteoporosis, osteoarthritis, and scoliosis. Methods: 2) Geniad mice will be screened by conventional X-ray for dental, skeletal, scoliosis and kyphosis phenotypes, 2) Micro-CT analysis of dental phenotypes, 3) Mapping of QTL to verify candidate genes for dental phenotypes, 4) *In vitro* gene expression and bioinformation analyses of candidate gene involvement. Results: X-ray screening of 1137 Geniad mice across 84 strains: scoliosis phenotype 4.3%, kyphosis phenotype 5.2%, kyphoscoliosis phenotype 1.7%, dental phenotype 21.6% (including hypodontia 7.03%). Conclusions: X-ray screening results approximate expected findings and indicate the need for micro-CT analysis, identification and mapping of QTL, and determination of candidate gene involvement for dental, scoliosis and kyphosis phenotypes.

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Carbamazepine does not rescue the osteogenesis imperfecta bone phenotype in *Col1a2*$^{+/G610C}$ mice but increases fragility in healthy bones

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The most common osteogenesis imperfecta (OI) mutations are in the collagen I genes, *COL1A1* and *COL1A2*. The mutations cause collagen misfolding and intracellular retention, and ultimately, bone matrix defects. In a mouse chondrodysplasia model

caused by a collagen X misfolding/aggregation mutation, stimulating autophagy and proteasomal degradation pathways with the drug carbamazepine (CBZ) reduced both the protein aggregates and the dwarfism severity. CBZ is now in clinical trials for collagen X chondrodysplasia. We reasoned that CBZ might improve OI through a similar mechanism, so we tested CBZ treatment in the *Col1a2*$^{+/G610C}$ OI mouse model, in which collagen I misfolding and intracellular retention underlying pathology.

Three week old male *Col1a2*$^{+/G610C}$ mice and wildtype littermates were gavage-fed CBZ for 3 weeks (escalating to 250 mg/kg/day) followed by a 3-week CBZ slow release implant (250 mg/kg/day).

Micro-computed tomography showed *Col1a2*$^{+/G610C}$ femora were 11% narrower than wildtype littermates. While CBZ did not increase bone width in *Col1a2*$^{+/G610C}$ mice, it significantly reduced bone width (by 7%) in wild type mice.

In 3-point-bending tests, *Col1a2*$^{+/G610C}$ bones were ~60% weaker than wildtype bones. CBZ failed to improve this. Instead, CBZ-treated wildtype bones were ~25% weaker than wildtype controls. This was explained by the narrower width, not by material quality, since no strength defect was present when corrected for bone size. This indicates that CBZ increases bone fragility in wildtype mice by limiting bone growth.

Bone composition assessment by Fourier transform infrared spectroscopy confirmed a greater mineral:matrix ratio in *Col1a2*$^{+/G610C}$ bones than wildtypes. CBZ did not affect wildtype or *Col1a2*$^{+/G610C}$ bone composition.

These data indicate that CBZ does not improve bone mass, strength or quality in *Col1a2*$^{+/G610C}$ osteogenesis imperfecta. However, CBZ increases fragility in healthy bone by suppressing bone growth. This suggests CBZ treatment could negatively impact bone growth in children taking CBZ as an anti-epileptic medication.

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Higher levels of calciprotein particles are associated with reduced bone mineral density in patients with Fabry disease

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Background: Fabry disease (FD) is a genetic disorder caused by mutations in GLA, the X-linked gene encoding α-Galactosidase A. Deficient enzyme activity leads to the build-up of lysosomal contents, contributing directly or indirectly to multiple pathologies centered mostly on the kidney, heart, and central nervous system, but patients often also experience osteopenia or osteoporosis.

The liver-derived glycoprotein fetuin-A stabilizes calcium and phosphate in extracellular fluid by forming colloidal mineral-protein complexes, calciprotein particles (CPP), which facilitate the stabilization, transport and clearance of excess mineral from the circulation. While most CPP circulate as monomers, they combine to form spheroidal primary CPP (CPP-I) and larger, elongated secondary CPP (CPP-II), both of which are associated with vascular and renal pathology when present at high levels.

Methods: We measured the serum CPP-I and CPP-II levels of 59 individuals with Fabry disease (63% female; mean age 45 ± 13) by flow cytometry and compared them to measurements of bone mineral density (BMD) and serum biochemistry, adjusting for demographics, comorbidities, and medications.

Results and Conclusions: We found that low BMD at the total hip and femoral neck was associated with high levels of CPP, in particular CPP-II (Table 1), and this relationship was weakly associated with male sex but independent of kidney function. This is the first time sex differences in CPP levels have been observed, and it is probably due to the X-linked nature of the disease, since both low BMD and high CPP may be markers of more severe disease in these individuals. The co-occurrence of these phenomena implies a direct relationship linking the bone and CPP systems, which merits further investigation to establish if it is unique to FD or a more generalized association.

Table 1: Association between bone mineral density and serum calciprotein particles in patients with Fabry disease

Skeletal site (n)	Serum CPP-I[a]		Serum CPP-II[a]	
	β[b]	P value	β[b]	P value
Lumbar Spine (g/cm^2) (55)	-0.07	0.614	-0.18	0.191
Lumbar Spine Z score (59)	-0.10	0.444	-0.13	0.304
Lumbar Spine T score (58)	-0.08	0.552	-0.19	0.159
Total Hip (g/cm^2) (55)	-0.22	0.101	-0.30	0.0234
Total Hip Z score (57)	-0.27	0.043	-0.29	0.028
Total Hip T score (56)	-0.29	0.032	-0.37	0.005
Femoral Neck (g/cm^2) (55)	-0.25	0.064	-0.32	0.017
Femoral Neck Z score (55)	-0.30	0.028	-0.30	0.024
Femoral Neck T score (54)	-0.30	0.028	-0.38	0.004

[a] natural log transformed

[b] standardised correlation coefficient

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Preptin deficiency has sexually dimorphic effects on trabecular bone volume in mice with advancing age

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Preptin is a 34-amino acid peptide derived from the E-peptide of pro-IGF-II. Preptin is co-secreted with insulin from β-cells, can increase glucose-stimulated insulin secretion, and promotes proliferation and differentiation of osteoblasts. We tested the hypothesis that preptin deficiency alters bone metabolism by evaluating a preptin knockout (KO) mouse.

Experimental KO and wild type (WT) mice were generated by heterozygous breeders. Adult livers (n=4-9) had similar *Igf2* mRNA expression between genotypes, with undetectable preptin expression in KO mice. Metabolic phenotypes were evaluated by weekly fasting blood glucose measurements, intraperitoneal insulin tolerance tests (ITT) at 9, 29, and 44-weeks of age, and oral glucose tolerance test (GTT) at 45-weeks of age (n=12-14/sex/genotype). Bone phenotypes were evaluated by femoral microCT at 6-weeks (n=8-12/sex/genotype; immature), 14-weeks (n=10-12/sex/genotype; peak bone mass), and 47-weeks of age (n=12-14/sex/genotype; aging).

Bodyweights were similar between genotypes at all ages. Blood glucose concentrations returned to baseline quicker following ITT in female KO than WT mice at 9-weeks of age only. Female KO had increased blood glucose concentrations 15- and 30-minutes post-glucose during GTT compared to WT mice. There were no metabolic differences in males.

There were no differences between genotypes in bone microarchitecture at 6-weeks of age. By 14-weeks of age, trabecular bone volume fraction (BV/TV) was increased by 21%, trabecular number was increased 17%, and cortical bone area was increased 8% in male KO vs. WT mice. These effects were absent in females. At 47-weeks of age, males had similar bone microarchitecture; however, trabecular bone volume fraction was increased by 29% (p=0.09), and trabecular number was increased by 30% in female KO vs. WT mice.

Male preptin KO mice had increased trabecular bone volume at 14-weeks of age only, whereas female preptin KO mice developed this phenotype as they aged. Mechanistic evaluation of this phenotype is ongoing.

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Silver nanoparticles as an antimicrobial and their effects on osteogenic cells and bone regeneration

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Introduction

The antimicrobial properties of silver are well known. Silver nanoparticles (AgNPs) are increasingly being investigated as a non-antibiotic alternative for bone grafting. This research investigated the potential cytotoxicity and intra-cellular uptake and gene expression of bone related cells exposed to AgNPs.

Methods

Lipoic-capped-AgNPs were synthesised and assessed via inductively coupled plasma-mass spectrometry and TEM. Osteoblast (Saos-2) and osteoclast (RAW 264.7) cells were tested with AgNPs from 0.0225-50 µg/ml with controls of carrier only, chlorhexidine digluconate and silver nitrate for cell viability and IC50's calculated. TEM was conducted without heavy metal staining to preserve the AgNPs. Gene expression assays with qRT^2-PCR were undertaken on osteoblasts for stress-related genes and a STRING analysis presented.

Results

AgNPs were generated with a mean hydrodynamic size of 7.5 nm. Cell viability assays showed osteoclasts were more susceptible to AgNPs than osteoblasts. TEM found AgNPs both as nano-particles and nano-chain assemblies within the cytosol of cells. AgNPs at 10 µg/ml for 48 h on osteoblasts activated a process of autophagy into vacuoles and autolysosomes. There were 28 significantly regulated genes with HMOX1 (86-fold upregulation) as a hub gene, and the only gene regulated at 4 h with 1 µg/ml of AgNPs.

Conclusion

AgNPs on osteoblasts and osteoclasts causes cytotoxic effects with particles sequestered within cells via autophagy. The gene expression prolife suggests the osteoblasts work to suppress reactive oxygen species levels as a survival mechanism and that autophagic cell death is active as a response to environmental stress. HMOX1 acts as a protective anti-apoptotic protein and its early regulation indicates cells rapidly activate protective pathways. Osteoclasts were particularly suspectable to AgNPs, which they internalise as nano-chains or aggregates. AgNPs differential effect on bone cells may provide an antimicrobial environment with preference to osteoblasts over osteoclasts.

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The Needle Insertion Surgical Model with *Staphylococcus aureus* Biofilm and Orthopedic Implant for Preclinical Drug Trials

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Introduction: Osteomyelitis remains a major clinical challenge in orthopaedics. It is particularly demanding when the infection is associated with a bacterial biofilm and/or antimicrobial drug resistance. Reliable and cost-effective preclinical models are essential for testing new interventions. Prior published rodent fracture infection models employed rats, which are costly compared to murine models.

Aim: To develop a dependable and cost-effective murine bone infection model with a needle insertion surgery (NIS) that mimics bacterial bone infections associated with biofilm and metal implants.

Methods: A metaphyseal bone infection model with tibial drilled hole (TDH) and needle insertion surgery (NIS) were compared in C57BL/6 mice (female, N=80). Metal pins were inserted selectively into the medullary canal adjacent to the defect sites. A free *Staphylococcus aureus* (ATCC-12600) or biofilm (ATCC-25923) suspension was locally inoculated (10^5 CFU in 5 µL). Animals were monitored for physiological or radiographic evidence of infection without prophylactic antibiotics for up to 14 days. At the endpoint, bone swabs, soft-tissue biopsies and metal pins were taken for bacterial culture. X-ray and micro-CT scans were performed along with histology analysis.

Results: TDH and NIS methods achieved a 100% success rate of infection in tibiae when a pin was present with free bacteria injection. NIS is a faster and less complex procedure than TDH, causes less physical disability to the animals. A biofilm inoculation alone induced 40-50% of infections without a metal implant, significantly higher than free bacteria (30%).

Conclusions: To reliably create progressive osteomyelitis, either a metal surface permissive for biofilm formation needs to be present, or defects are inoculated with an established biofilm. The NIS method is a practical approach to produce a bone defect suitable for modelling surgery-related osteomyelitis. Subsequent studies will apply these preclinical models to trial antimicrobial therapies.

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Time-lapse quantitative characterisation of whole-joint morphometric changes in a collagenase-induced osteoarthritis mouse model

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Osteoarthritis (OA) is a progressing and complex disease that causes structural changes to the entire joint. Recent studies using 3D quantitative morphometric analysis (QMA) have shown that *in situ* micro-computed tomography (microCT) imaging can quantify structural changes of the whole joint in rabbit and rat anterior cruciate ligament transection models of OA [1,2]. Collagenase-induced OA (CIOA) is another OA model, and several studies observe structural changes related to disease. However, no previous research has measured these changes with disease progression nor looked at whole-joint morphometric changes in 3D. Forty-eight 10-week-old male C57BL/10 mice underwent OA induction via intra-articular collagenase injection at the knee, and a further 24 mice served as age-matched controls. Each week following the injection (up to 8-weeks), 9 mice (3 controls, 6 OA) were sacrificed, then scanned using microCT (vivaCT80, Scanco Medical). Scans were performed with a nominal voxel size of 10 µm. QMA measurements of joint centre of mass, as well as medial and lateral joint space width (JSW_M, JSW_L) and volume (JSV_M, JSV_L) were calculated. A two-factor ANOVA was used to determine the effect of time and disease. Results show that CIOA caused significant shift of the joint centre of mass in anterior/posterior and superior/inferior directions, along with increases in JSW_M and JSW_L, which were not affected by time. Meanwhile larger JSV_M and JSV_L, driven by both disease and time, were observed in CIOA samples from week 7. Typical joint space for each week is shown in Figure 1. These findings provide a preliminary insight into how CIOA causes structural change that should be further interrogated to identify links between biochemical and biomechanical triggers. Evaluation of histological data and semi-quantitative scores are planned, as well as repeating the experiment with more samples to identify statistically significant changes in morphometric markers linked to the

CIOA model.

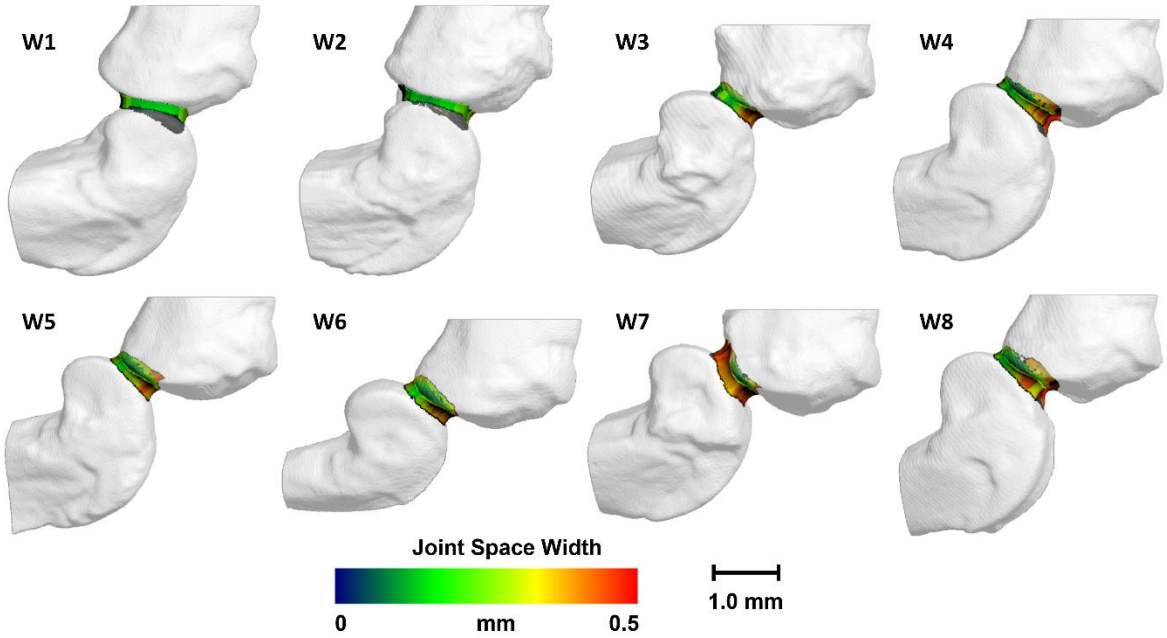

Figure 1: Typical visualisations of the joint space width of the mouse right knee's medial compartment for each week after CIOA was induced.

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Morpho-molecular and biochemical assessment indicates loss of calcified cartilage integrity in osteoarthritic joints

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The osteochondral interface is a thin layer in adults connecting hyaline cartilage and subchondral bone. The osteochondral interface undergoes significant changes during osteoarthritis (OA) progression. Previous studies mainly focus on cartilage and subchondral bone but underestimated the function and changes of osteochondral interface. The morphological, molecular, and biochemical changes of the osteochondral interface have not been fully understood yet. The aim of this study is to investigate the morpho-molecular changes of the osteochondral interface during OA progression. Based on the OARSI grading, the G1 and G4 knee osteochondral interface samples were selected from the medial and lateral parts of the tibial plates from 6 patients undergoing total knee replacement, and 10 medial tibia plates from rats undergoing sham or meniscectomy surgery, respectively. H&E staining, Safranin-O staining, immunohistochemistry, Scanning electronic microscopy, Energy-dispersive X-ray spectroscopy, Transmission electron microscopy, Fourier-transform infrared spectroscopy, Nanoindentation, Laser capture microdissection assisted proteomics were used to explore the characteristics of the osteochondral interface. Our results demonstrated that the osteochondral interface undergoes significant morphological and molecular changes, including the thinning of the calcified cartilage zone, loss of collagen and proteoglycan, occurrence of the endochondral ossification and neuro-vasculature, loss of the elastic module, loss of the collagen direction, increase of the tortuosity and the change of the protein expression in the region. The calcium/Phosphate ratio was not changed during the OA progression, but the calcium-binding protein and cadherin binding protein, as well as carbohydrate metabolism-related proteins, undergo significant changes during OA progression. These results suggest that the osteochondral interface undergoes significant changes driven by the changed expression of the proteins in this zone. Alleviating or reversing the pathological changes osteochondral interface could be conducive to treating OA.

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Identification of epigenetic factors deregulated in skeletal stem cells during high fat/glucose mediated inhibition of bone formation.

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Individuals with type 2 diabetes are at higher risk of osteoporosis and major bone fractures independent of their body mass index (BMI). Bone marrow derived mesenchymal stem/ stromal cells (BMSC) that are obtained from these patients show reduced osteogenic potential and increased cell death. Currently, it is not known how high fat/glucose levels can suppress BMSC osteogenic differentiation potential however we envisage that diet impacts epigenetic regulators in BMSC. The present project examined the epigenetic mechanisms regulating BMSC dysfunction in response to high fat/glucose to identify targets for reversing high fat-mediated bone loss, aging and diseases. We hypothesize that high fat/glucose levels lead to changes in epigenetic gene expression patterns in BMSC that leads to compromised bone formation, which increases the likely hood of osteoporosis. Ten-eleven translocation (Tet) family is a group of DNA demethylases, able to convert 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), an epigenetic marker in osteogenesis. Our studies have shown that Tet2 is essential in driving the differentiation of bone forming osteoblasts and high glucose level inhibits the expression of Tet2. Furthermore, human BMSC grown in high glucose conditions were found to increase cell death, senescence and oxidative stress while decreasing cellular proliferation potential compared to regular growth media. High glucose levels also were shown to drive the differentiation of BMSC towards lipid forming adipocytes at the expense of mineral forming osteoblasts which was confirmed by differentiation gene expression analysis. Understanding the role of epigenetic regulators in hyperglycaemic conditions will help to identify solutions to battle bone loss seen in diseases such as diabetes and osteoporosis. Given that epigenetic marks can be reversed by pharmacological inhibitors and altered via changes in diet and lifestyle, these targets are of unique therapeutic importance.

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Mass spectrometry tissue imaging identifies complex/hybrid-type *N*-glycans as putative novel cartilage degradation markers for human knee osteoarthritis

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Objective:

Knee osteoarthritis (KOA) is the most common form of arthritis, but the biomolecular, especially post-translational modifications such as *N*-glycans, involvement of its onset and progression is controversial. Thus, the aim of this study was to spatially localise, identify, and compare *N*-glycans from formalin-fixed paraffin-embedded (FFPE) osteochondral tissue in KOA patients and cadaveric controls (CTL).

Methods:

FFPE osteochondral tissue from end-stage KOA patients (n=3) and CTL individuals (n=3), >55 years of age, were analysed by advanced matrix-assisted laser desorption/ionisation mass spectrometry imaging (MALDI-MSI). *In-house* developed gelatin pre-coating workflows were used to obtain the imaging data. Based on the theoretical masses, *N*-glycan peaks were then manually selected, and ion intensity maps were generated using FlexImaging and SCiLS Lab software. Putative *N*-glycan structures were annotated using the following tools: GlycoMod, which calculates the theoretical monosaccharide composition, and Glycoworkbench to create individual *N*-glycan structures.

Results:

MALDI-MSI revealed differential *N*-glycan profiles between KOA patients and CTL individuals within the cartilage region only. Overall, 26 *N*-glycans were found significantly elevated in KOA cartilage as compared to CTL cartilage, with approximately a 2.5-fold increase in the signal intensity. In addition, there were particular three complex/hybrid-type *N*-glycans of *m/z* 1298.4, 1501.5, and 1663.5 ± 0.5 Da found predominantly in the upper fibrillated surface of degraded cartilage (OARSI histological grade 2.5-3), with minimal signal intensity in the adjacent surface with less damaged cartilage (OARSI histological grade 1-1.5).

Conclusion:

Our preliminary results demonstrate the novel application of MALDI-MSI to identify and localise KOA cartilage-specific *N*-glycans. The alterations of these particular complex/hybrid-type *N*-glycans could evolve into a potential cartilage degradation marker and a possible new target for future treatment of cartilage degradation in patients with KOA. Further validation of these results is currently in progress using a fragmentation technique called liquid chromatography/tandem mass spectrometry (LC-MS/MS).

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Three-dimensional quantitative morphometric analysis (QMA) for longitudinally tracking tibiofemoral microstructure in a preclinical mouse model

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Osteoarthritis (OA) is a degenerative joint disease, causing structural changes in the whole joint. Recent studies showed three-dimensional quantitative morphometric analysis (QMA) is a valuable tool to track joint change from micro-computed tomography (microCT) images. However, it has only been applied in *ex vivo* cross-sectional studies to date [1-2]. In this study, we tested the feasibility of an *in vivo* longitudinal protocol for evaluating joint and bone QMA in a preclinical mouse model, with a goal for future use in OA studies. The knees of four healthy C57Bl/10 mice were scanned weekly for 8 weeks using microCT (vivaCT80, Scanco Medical) at a nominal voxel size of 10 µm. 3D QMA was performed on the dataset to evaluate changes to epiphyseal trabecular and cortical bone, and the lateral and medial joint. These joint parameters include center of mass vector length λ, and orientation α, β, γ, lateral and medial joint space width JSWL, JSWM, and lateral and medial joint space volume JSVL, JSVM [1]. All joint parameters showed little deviation over time (Fig. 1). Specifically, λ was consistent in length and orientation, where most deviation is observed in γ, which is associated with joint flexion. Likewise, little change to epiphyseal trabecular bone (BV/TV, Tb.Th, Tb.N and Tb.S) and cortical bone (Ct.Th, Ct.Po) was observed. This study demonstrates feasibility of an *in vivo* longitudinal protocol

for evaluating joint and bone QMA in a preclinical mouse model. As expected in a skeletally mature animal, joint parameters are stable over time. Small deviations are expected and highlight the need for consistent positioning of the joint. Next steps will focus on applying the protocol to an OA dataset to reveal changes associated with disease progression, as well as histopathological analysis for validation.

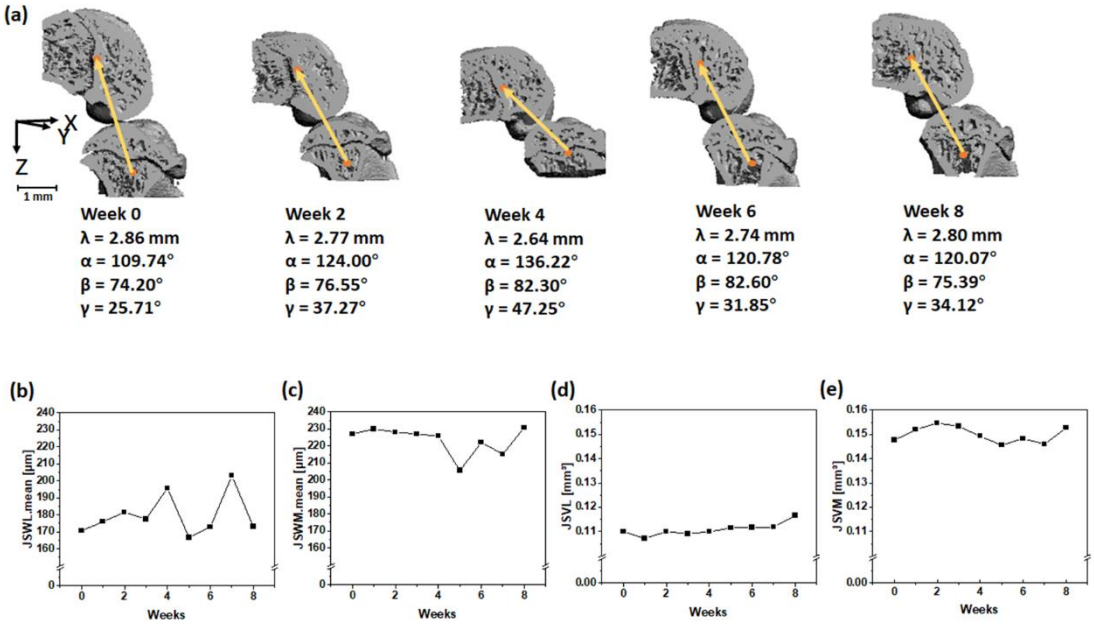

Figure 1. (a) 3D visualization of cortical and trabecular bone in a right knee joint over time, including 3D QMA joint parameters: center of mass vector length, λ (mm), and orientation α (°), β (°), γ (°). (b) Lateral joint space width (µm), (c) medial joint space width (µm), (d) lateral joint space volume (mm³), and (e) medial joint space volume (mm³).

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The role of skeletal muscle in maintaining vitamin D status

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The long residence time of 25(OH)D in blood of 50 days or more, suggests that there is some store of this metabolite, when there is no input of vitamin D from the environment. Yet no functional store has been found. There are several reports of higher blood concentrations of 25(OH)D in people exercising in winter, even indoors, compared to those who have a more sedentary lifestyle. This suggests that some function of skeletal muscle may have a role in maintaining vitamin D status. Muscle biopsies from sheep have significantly higher concentrations of 25(OH)D in winter than in summer. Studies with muscle cells *in vitro* have revealed the presence of megalin/cubilin proteins in the plasma membrane of these cells. These membrane proteins transport vitamin D-binding protein (DBP) from the extracellular fluid into the myocytes where some binds to actin and the rest remains free in the cytoplasm. This internalised DBP provides an array of high affinity binding sites for 25(OH)D which diffuses into the myocytes from the extracellular fluid. However, DBP in the muscle cell cytoplasm soon undergoes proteolysis. This then releases 25(OH)D which diffuses from the myocytes and returns to the circulation. With the seasonal fall of blood 25(OH)D in winter, some regulating signal is proposed to increase the DBP uptake and thus its concentration in skeletal muscle cells, so that 25(OH)D concentration in those cells also rises. These observations suggest that the repeated cycling of 25(OH)D into and out of skeletal muscle cells, accounts for its long residence time in blood. Variable regulation of intracellular DBP levels in myocytes would thus allow variable rates of cycling of 25(OH)D to and from the circulation with consequent variable residence time in blood. It is proposed that some feature of muscle undergoing regular exercise promotes this conservation mechanism for 25(OH)D.

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The role of vitamin D in the maintenance of locomotor function

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Skeletal muscle predominantly controls basal metabolism by sustaining locomotive function. Development and maturations of skeletal muscle functions are affected by vitamin D endocrine function, as skeletal muscle-specific vitamin D receptor (VDR) knockout (KO) mice exhibited losing both muscle mass and performances. However, there were few studies verified the timing when vitamin D action becomes more dominant during myogenic development. Therefore, we investigated the maturations of skeletal muscle cells isolated from muscle-specific VDR KO mice.

Muscle-specific VDR KO mice was obtained by crossing skeletal muscle specific cre (creatine kinase cre) mice with VDR flox mice. A lack of VDR activity decreased locomotor performance measured by latticed infrared beam interruptions in the cage. As bone phenotype, the increased osteoclast activation appeared in these mice and resulted to the reduction of bone mass. Since bone metabolism participates to the muscle development, $1,25(OH)_2D_3$-VDR activity in myoblast differentiation was assessed in cultured C2C12 cells in next step. During differentiation, the levels of VDR protein was higher in the early stage compared to the mature stage. To analyze ATP metabolism, protein expression of both CX43, a promoter of ATP transport, and ENPP1, an eliminator of ATP, were evaluated. The level of CX43 was decreased by cellular differentiation while ENPP1 was increased. Furthermore, the ratio of ATP/ADP at every 24-hour in cultured skeletal muscle was increased by $1,25(OH)_2D_3$ stimulation in dose-dependent manner, the maximal effect was observed in 10^{-12}M.

In conclusion, current study indicated that local VDR activity regulate the supply and the elimination of ATP in skeletal muscle, which is essential for muscle contraction, thereby contributes the physiological controls of locomotive function.

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Generating a post-natal knockout of sclerostin using adeno-associated viruses

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Background: Generating controlled spatial and temporal knockouts in mice is a lengthy and expensive process. In bone, it is also often hindered by issues of embryonic lethality and confounding phenotypes in other tissues. Recombinant adeno-associated viral vectors (rAAV) offer an alternative method for gene editing applicable to neonatal and adult mice. Our group has previously produced a bone-specific rAAV (AAV8-Sp7-Cre) that demonstrates high efficiency and specificity for osseus tissues. It was hypothesized that this AAV-Cre vector could be employed to create targeted gene disruption in postnatal animals. This study aimed to knockout the murine Sclerostin (*Sost*) gene, previously shown to be a key negative regulator of bone mass.

Methods: 8-week-old $Sost^{flox/flox}$ mice were systemically injected with either AAV8-Sp7-Cre at $5x10^{11}$ vg/mouse or saline. Bone density was measured by longitudinal DXA for 6 weeks. Fore and hind limbs and vertebrae were harvested for genetic analysis, microCT, biomechanical testing, standard bone histology and dynamic histomorphometry.

Results: $Sost^{flox/flox}$:AAV-Cre mice showed enhancement of numerous bone parameters including trabecular and cortical thickness, trabecular bone volume fraction, and cortical bone volume. This was associated with a +25% increase in mineral apposition rate in the cortical bone. Analysis of AAV8-Sp7-Cre mediated recombination in an Ai9 fluorescent reporter mouse model confirmed efficient and bone-targeted vector activity.

Discussion: Consistent with prior reports of *Sost* null mice, $Sost^{flox/flox}$:AAV-Cre demonstrate a high bone mass phenotype and increased bone anabolism. This technology represents a new and streamlined approach to generate conditional gene knockout mice restricted to postnatal bone, where floxed mouse strains are available. This technology is not only economical and versatile, but it also overcomes challenges with studying genes where developmental disruption is embryonically lethal.

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BIOMECHANICAL DETERMINANTS OF JAW FRACTURE AND REPAIR

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Choosing the best technique to fix a broken jaw is an important topic in oral and maxillofacial surgery and there is ongoing debate amongst surgeons about whether non-rigid or rigid fixation is the best method of treatment[1-5]. We have some understanding of the mechanics of the healthy mandible during feeding[6-10], and the impact of fracture fixation technique on static bite forces[11-14], but we do not know how treatment rigidity alters bone strain regimes during chewing. Hence not only do we not know which fixation treatment results in better healing, we do not have a body of biomechanical theory to explain why one treatment modality is better. Knowledge of the abnormal strain environments —strain regimes to which bones are not adapted— in and around the fracture zone and implants is important if, abnormal strain environments can stimulate post-operative complications such as sub-optimal bone (re)modelling, resulting in weak bone morphology, slow healing, and mal-unions[15].

This study is the first to use realistic simulations to compare the biomechanical behaviour of the human and macaque mandibles pre and post angle fracture and fixation. We show that macaques are ideal animal models for oral and maxillofacial research

because they chew like humans. We also show that non-rigid fixation results in higher strains in the bone-implant interfaces and a higher degree of interfragmentary displacement than rigid fixation (**Figure1**). One of the most salient results of our study is the importance of laterality of chewing behaviour post treatment. Chewing contralateral to the fracture increases interfragmentary displacement beyond the optimal threshold suggested by orthopaedic literature (**Figure1**). This shows that non-rigid fixation and contralateral chewing likely inhibit bone healing and accelerate post-operative complications. Future research combining in vivo experiments, biomechanical modelling and histology will further elucidate the links between treatment rigidity and bone healing in the mandible.

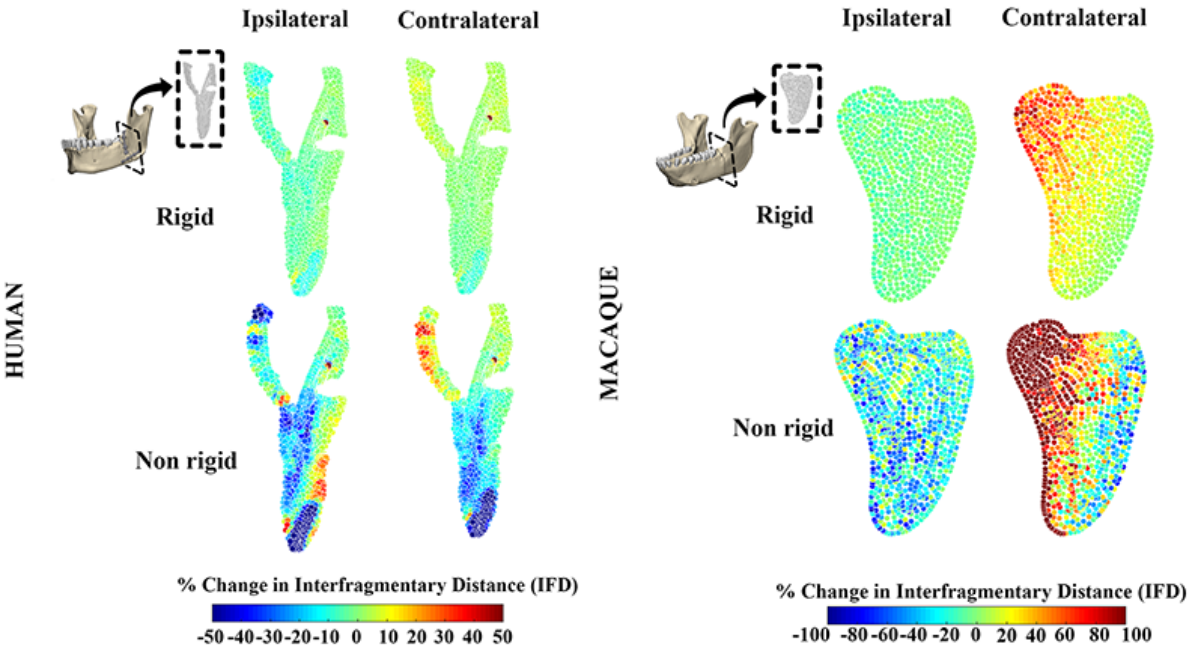

Figure 1. % change in interfragmentary distance (IFD) between nodes across the fracture plane during chewing ipsi- and contralateral to the fracture in macaques and humans. Warm and cold colors show areas with high and low IFD. **Note** the increase in IFD under the non rigid treatment and contralateral chew.

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PATHOGEN ASSOCIATED MOLECULAR PATTERNS AFFECT NEUROGENIC HETEROTOPIC OSSIFICATION VIA SKELETAL MUSCLE CELLS

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INTRODUCTION Neurogenic heterotopic ossifications (NHO) are abnormal development of heterotopic bones in periarticular muscles after spinal cord injury (SCI), traumatic brain injury (TBI), or cerebral stroke. NHO causes joint ankylosis and compromises the quality of life for these patients. Retrospective studies in SCI and TBI patients suggest that infections are a significant risk factor of developing NHO. Our initial experiments show that mesenchymal fibro-adipoprogenitors in the muscles are the cells-of-origin of NHO. We have previously developed a mouse model in which NHO develops in injured hamstrings after SCI. In this model, we investigated whether pathogen-associated molecular patterns (PAMPs) from microbes, have the potential to exacerbate NHO.

METHODS Through osteogenic assays, purified PAMPs were added in cultures of sorted muscle (FAPs), to test the potential of PAMPs to enhance mineralisation, both directly and indirectly via macrophages, which are essential drivers of NHO. After 10-14 days, calcium deposition was measured with alizarin red staining. For in vivo experiments, C57BL/6 mice underwent SCI, and PAMPs were co-administered with cardiotoxin (CDTX) in hamstring muscles. NHO volumes in injured muscles were measured via µCT at day 7-21 post-injury.

RESULTS In vitro, PAMPs such as Pam2CSK4, Pam3CSK4 and Zymosan induced a dose-dependent increase in FAP mineralisation. We also observed that all PAMPs increased the mineralisation potential of FAPs indirectly. Supernatants from bone marrow-derived macrophages cultured in the presence PAMPs could promote FAP mineralisation in vitro. In vivo, co-administration of lipopolysaccharides (LPS) or lipoteichoic acid (LTA) with cardiotoxin significantly increased NHO volumes. Interestingly, PAMPs such as zymosan and gardiquimod significantly decreased NHO formation.

CONCLUSION We have established that PAMPs could increase the mineralization capacity of FAPs. Some bacterial PAMPs exacerbated NHO when injected in the injured muscles. Future investigations are warranted to determine whether intramuscular injection of zymosan and gardiquimod deplete/compromise macrophages in injured muscles.

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Micro-computed tomography reveals early bone structural changes in osteoarthritis progression in a collagenase-induced mouse model

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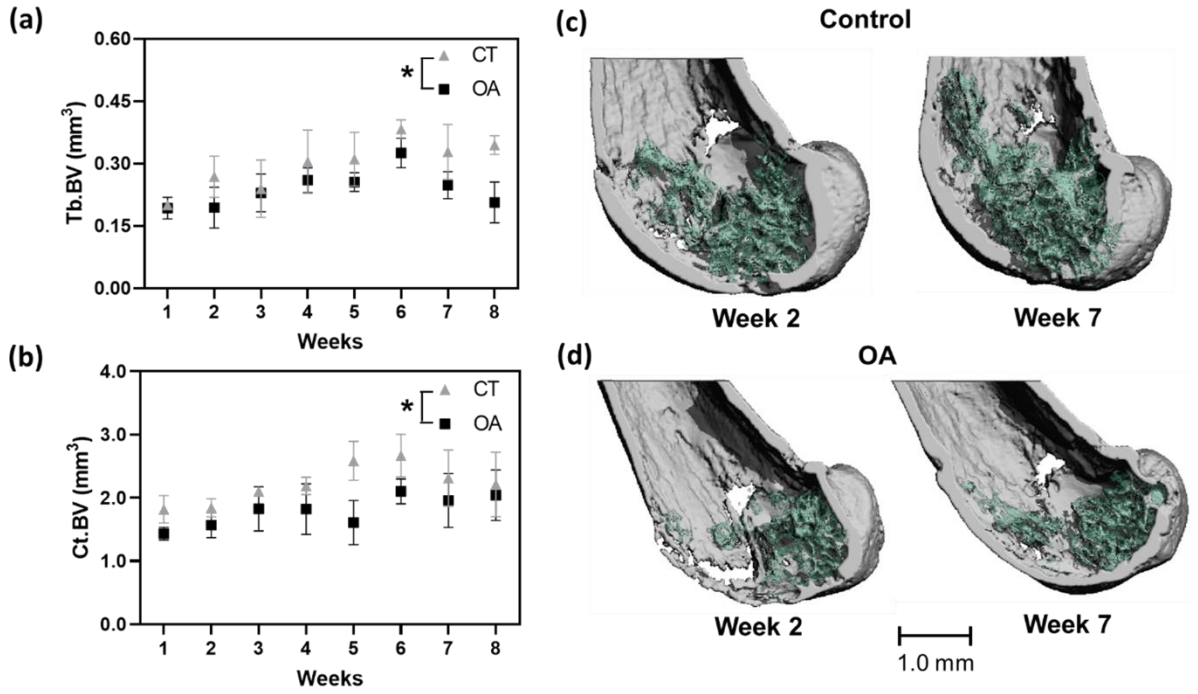

Figure 1 *Lateral femoral (a) trabecular bone volume (Tb.BV) and (b) cortical bone volume (Ct.BV) from samples scanned in weeks 1-8. MicroCT images of (c) control samples, and (d) OA samples from weeks 2 and 7. Green and grey represent the trabecular and the cortical regions, respectively.*

Osteoarthritis (OA) is associated with tissue damage and loss of function, which can be observed in preclinical studies. One OA mouse model, a single intra-articular injection of bacterial collagenase, can lead to matrix depletion in the femorotibial joint. An acute inflammatory response starts immediately; however, onset and progression of structural changes has not been described. The aim of this study was to investigate bone structure changes during OA progression using a collagenase-induced OA mouse model and micro-computed tomography (microCT). A single intra-articular injection of bacterial collagenase solution was used to induce OA in the knee joint of C57Bl/10 mice (n=48 OA and n=24 controls). Mice were culled weekly (6 OA, 3 control) and scanned with microCT (vivaCT80, Scanco Medical, 10µm, 70 kVp; 57 µA) for 8 weeks (72 scans). Bone morphometry analysis was performed to quantify subchondral trabecular and cortical bone. Three-factor ANOVA was performed to determine the effect of time (weeks), site (lateral, medial) and group (OA, control). Bone volume (Tb.BV) of the femoral trabecular region was generally lower in OA samples than controls, and significantly lower in weeks 2, 4, and 7 (Figure 1a). In week 7 and 8 there is a noticeable downward shift in OA bone volume. This is linked to a higher trabecular spacing and lower trabecular number (data not shown). The same trend was observed in both medial and lateral compartments. The femoral cortical region showed significant differences from week 1, e.g. lower Ct.BV (Figure 1b) in OA samples compared to controls. These differences are visible in microCT images of typical control (Figure 1c) and OA samples (Figure 1d) from weeks 2 and week 7. These findings imply bone remodelling activity occurs simultaneously with the acute inflammatory response in this model, which will be more thoroughly investigated in follow-up studies.

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Deregulation of DNA hydroxymethylases Tet1 and Tet2 compromises skeletal integrity during ageing and osteoporosis

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Bone mesenchymal stem/ stromal cells (BMSC) reside in bone marrow and can give rise to several cell lineages, including lipid forming adipocytes, cartilage forming chondrocytes and bone forming osteoblasts. Osteoblastic differentiation, driven by *Runx2,* is a key pathway for the development and maintenance of healthy bone tissue. Altered expression of osteogenic associated genes can lead to bone deficiencies such as Type I and II osteoporosis, resulting in a higher incidence of major fractures. While a common pathology, only 10% of the genetic risk factors for osteoporosis have been uncovered, implying that epigenetic deregulation may be a crucial component. The DNA hydroxymethylases, Tet1 and Tet2 are epigenetic modifiers that have been found to be downregulated during osteoporosis, implicating Tet molecules in the onset and progression of osteoporosis. Investigation into the roles of Tet1 and Tet2 during bone development, as well as the changes in genome wide occupancy of Tet molecules and methylation status of key osteogenic genes during ageing were assessed using conditional double knockout (DKO) of Tet1 and Tet2 in Prrx-1 positive BMSC. Analysis of bone parameters including bone and tissue volume, trabeculae size and osteoblast/ osteoclast number were assessed by 3D mCT and histomorphometric analyses, at various ages (embryonic, new born, 1, 3 and 12 months) to determine the effects of Tet1 and Tet2 on bone density and integrity over time.

Preliminary studies found a decreased bone volume and changes in trabecular number in 3 month old Tet 1 and Tet2 DKO animals compared to controls, with the effect being most prevalent in males. Further elucidation of the mechanisms driving Tet1 and Tet2 regulation of bone formation and maintenance may help develop new prognostic indicators of fracture risk and therapeutic strategies to reverse bone loss during ageing.

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Heterogeneity and its hierarchy of Prrx1-positive cells during limb development

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Paired related homeobox 1 (Prrx1) is expressed in mesenchymal progenitors in the limb buds and craniofacial mesenchyme. The mouse lineage tracing study indicates that Prrx1-positive cells enable to produce the chondrocytes, osteoblasts, bone marrow stromal cells, tendon/ligament cells and dermal fibroblasts during the development of limb skeleton. Here we use the single-cell RNA sequencing (scRNA-seq) to dissect the heterogeneity of Prrx1-positive cells and hierarchy of its descendant cells. Limb from each fetal stage (E12.5, E14.5, E16.5) of Prrx1 transgenic GFP mice, in which GFP is expressed under the control of the Prrx1 promoter, were single-celled by collagenase treatment, and then Prrx1-GFP-positive/negative cells were isolated by FACS. These cells were subjected to scRNA-seq by the microwell-cased scRNA-seq technology (BD Rhapsody). We have captured about 20,000 single cells, and identified 20 subpopulations highlighting the heterogeneous nature of the limb development. Our scRNA-seq studies provide a detailed molecular information that patterns limb skeleton.

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Foxf2 represses bone formation via canonical Wnt signaling

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【Introduction】

Differentiation of mesenchymal stem cells (MSCs) into osteoblasts is an essential process for the acquisition and maintenance of bone mass. However, its regulatory mechanism remains unclear. In this study, we focused on the forkhead transcription factor *Foxf2,* whose role in bone remodeling is still unknown. Thus, this study aimed to elucidate the regulatory mechanism of MSCs differentiation into osteoblasts by Foxf2.

【Methods】

Foxf2 was overexpressed or knockdown in mouse bone marrow-derived mesenchymal stem cell-like ST2 cells, and the expression levels of osteogenic differentiation markers were measured by quantitative RT-PCR. Then, we generated MSC-specific Foxf2 knockout mice (cKO mice) using Prx1 promoter and analyzed bone volume by μCT. Furthermore, we analyzed the target genes of Foxf2 by RNA-seq, Western blotting, and reporter assay. Finally, the in vivo effect of Foxf2 knockdown was evaluated by μCT in the bone marrow ablation model.

【Results】

Overexpression of Foxf2 in MSCs inhibited their differentiation into osteoblasts, while knockdown of Foxf2 promoted their differentiation into osteoblasts. cKO mice showed no obvious abnormality in bone resorption but enhanced bone formation, resulting in a high bone mass phenotype. Molecular biological analysis revealed that Foxf2 regulates the differentiation of MSCs into osteoblasts by modulating the Wnt pathway. In addition, Foxf2 knockdown promoted bone regeneration in vivo.

【Discussion/Conclusion】

Foxf2 regulates the differentiation of MSCs into osteoblasts via Wnt signaling. From a clinical point of view, the regulation of Foxf2 expression may be useful in promoting osteogenesis.

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Investigating bone health in high-yielding dairy cattle using biochemical markers of bone turnover, micro-CT and dynamic histomorphometry

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In dairy cattle, selection for high milk yields creates significant metabolic demand for all nutrients including calcium which is met by changes in bone homeostasis. The goal of the present study was to monitor the dynamics of bone turnover at different stages of lactation in a herd of high yielding dairy cows. Blood and milk samples were collected from 42 Holstein Friesian cows during late pregnancy (LP), early lactation (EL), mid lactation (ML), late lactation (LL), extended lactation (ExL) as well as non-pregnant, non-lactating young (heifers) and older (empty) cows. Samples were assayed for the bone resorption marker, CTX-I, and bone formation marker, osteocalcin. Rib biopsies were obtained from 33 cows during EL, ML, LL and LP. Biopsy samples were evaluated using micro-CT and dynamic histomorphometry. Generalised mixed model was used for statistical analysis in R. CTX-I concentration was decreased in LL, ML and ExL compared to baseline category EL. Some evidence was obtained that CTX-I was higher in heifers than during EL (Table 1). Small effects on serum osteocalcin levels were observed in cows at stages of lactation. Micro-CT evaluations showed total volume was higher in ML (β: 0.17, CI: 0.07-0.26) and LP (β: 0.28, CI: 0.13-0.41) as

compared to EL (β: 0) indicating a gradual increase in bone mass as milk production declines. Interestingly, the average total porosity was higher in ML 3.3% (CI: 2.8-3.8) and LP 3.5% (CI: 2.8-4.5) than in EL 2.8% (CI: 2.4-3.3) and LL 2.4% (CI: 1.9-3.0) cows, probably due to the porous nature of new bone. The data suggest that dairy cows adapt to the calcium demands of lactation by changing levels of bone resorption while maintaining constant bone formation. These findings can be used to further study how dairy management practices and breeding programs affect bone health in dairy cattle.

Table 1. Mixed Linear model describing effect of stages of production on CTX-I

	Coefficient	95 % Confidence Interval		p-value
		Lower	Upper	
Mid Lactation	-0.52	-0.89	-0.17	0.006
Late lactation	-1.03	-1.41	-0.64	$1.55*10^{-6}$
Extended Lactation	-0.84	-1.36	-0.32	0.002
Late Pregnancy	-0.16	-0.51	0.19	0.383
Empty	-0.01	-0.57	0.55	0.972
Heifers	0.30	-0.44	1.06	0.433
Early Lactation	0	—	—	—
Intercept	0.66	0.40	0.94	$4.25*10^{-6}$

R-squared marginal = 0.23 R-squared conditional = 0.33

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Unravelling osteoblast differentiation and function in myeloma: differences between lytic and non-lytic disease

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Publish consent withheld

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Identification of a Ferroptosis-Related Skeletal Dysplasia Spectrum and Its Potential Treatment

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Metaphyseal dysplasia is a phenotype often observed in various clinically skeletal dysplasia characterized by the shortening of the long tubular bones with viable severity. Many pathological mechanisms cause metaphyseal dysplasia. In this study, we recruited a series of skeletal dysplasia patients. The clinical diagnosis ranged from mild Patterson-Lowry rhizomelic dysplasia to Sedaghatian type spondylo-metaphyseal dysplasia which is lethal *in uterus*. By performing whole-exome sequencing analyses, we identified bi-allelic loss-of-function mutations of glutathione peroxidase 4 (GPX4) in eight patients from six pedigrees with multiple ethnic backgrounds.GPX4 is the only enzyme that can repair lipid peroxidation. It has been previously reported that insufficient GPX4 activity is the direct and major cause of ferroptosis, a type of programmed cell death characterized by the accumulation of lipid peroxides. Up-to-date, ferroptosis has only been only reported in organs with high oxygen-level and in common diseases. In this study, we proved that the pathogenic mechanism of the GPX4-related metaphyseal dysplasia was ferroptosis. We showed that ferroptosis could also take place in ultra-hypoxic organs such as the growth plate cartilage and could also be the cause of Mendelian diseases. Furthermore, we generated the cartilage-specific GPX4 conditional knockout mouse model. Our animal experiment showed efficient rescue effects by feeding the KO mice with Vitamin E, a natural antagonist of ferroptosis, indicating future treatment for the patients with GPX4 loss-of-function mutations.

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Potential regulatory roles of miRNA(s) in the bone loss and bone marrow adiposity following methotrexate chemotherapy in rats

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Methotrexate (MTX) is commonly used in cancer chemotherapy to treat childhood leukaemia and osteosarcoma. Although MTX chemotherapy improves the population of cancer survivors, the prevalence of chronic bone-related complications has increased. Reduced bone formation (osteogenesis) and increased marrow fat formation (adipogenesis) have been observed through a "switch-like" change in commitment of bone marrow stromal cells (BMSCs) following MTX treatment. However, the underlying molecular mechanisms of this bone/fat switch are not fully elucidated. MicroRNAs (miRNAs) participate in regulating BMSC differentiation by targeting osteogenesis/adipogenesis-related genes. Here, we found miR-6315 and miR-542-3p were differentially expressed in bone samples from MTX-treated rats. Target prediction tool and miRNA-mRNA network analyses indicated that Smad2 and Smurf2/sFRP-1 might be the direct target of miR-6315 and miR-542-3p, respectively. Subsequent luciferase assays confirmed the predictions. Additionally, *in vitro* cell models were applied to determine the potential roles of these miRNAs on osteogenic and adipogenic differentiation. Results suggest that miRNA agmoir supplement (miR-6315/miR-542-3p) enhanced osteogenesis, characterized by a significant increase in expression of osteogenesis markers RUNX2, ALP, OCN and OSX. On the other hand, miRNA agomir treatment inhibited adipogenesis and lipid droplet accumulation. Signalling pathway analyses demonstrated that miR-6315 can regulate bone/fat formation through the Smad2/TGF-β signalling and miR-542-3p can modulate bone/fat formation via the Wnt/β-catenin signalling. These findings may increase our understanding of how MTX damages the bone and may provide a foundation for further studies investigating potential bone damage biomarkers or therapeutic targets for patients who receive MTX chemotherapy.

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Association Between Tryptophan Metabolites, Physical Performance and Frailty in Older Persons

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Frailty is defined as a syndrome of physiological decline in late life, characterised by marked vulnerability to adverse health outcomes. A robust biomarker for frailty is still lacking. Tryptophan (TRP) metabolism through the kynurenine pathway (KP) plays essential roles in aging, the musculoskeletal system and physical performance. In this study, we quantified seven KP metabolites, including kynurenine (KYN), kynurenine acid (KYNA), quinolinic acid (QUIN), picolinic acid (PIC), 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HAA) and anthranilic acid (AA) using ultra-high-performance liquid chromatography and gas chromatography-mass spectrometry in the serum of 85 participants (median age 75; 65% female; 28 non-frail, 29 pre-frail, and 28 frail) at the Nepean Osteoporosis and Frailty (NOF) Study. We looked at the association between TRP metabolites and physical performance, sarcopenia, and frailty.

After adjusting for age and sex, our results showed that KYN and KYN/TRP were associated with higher IL6 levels (r=0.324 and r=0.390, respectively). KYNA and its ratios to other products (mainly KYNA/KYN, KYNA/QUIN and KYNA/PIC) were associated with lower likelihood of frailty by Fried's criteria (OR 0.93 [0.88, 0.98], p=0.009) and Rockwood index (r=-0.241, p=0.028) as well as lower likelihood of sarcopenia (OR 0.88 [0.78, 1.00], p=0.049). QUIN and QUIN/KYN showed an association with increased IL-6 (r=0.293 and 0.204 respectively), higher likelihood of frailty (OR 1.02 [1.00, 1.04], p=0.029 and OR 6.43 [2.23, 18.51], p=0.001 respectively) and lower physical function (r=-0.205 and r=-0.292).

In conclusion, different TRP metabolites have various associations with physical performance, frailty and sarcopenia. Defining the underlying mechanisms may permit the development and validation of new biomarkers and therapeutics for frailty and musculoskeletal conditions targeting specific metabolites of the TRP catabolic pathway

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Antipsychotic use and bone quality

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Background: Antipsychotic medication, used in the treatment of schizophrenia, has been shown to be associated with lower bone mass. However, less is known about the impact of antipsychotic medications on bone quality. Thus, we aimed to investigate the association between antipsychotic use and bone quality in a population-based sample of adults.

Methods: Data were collected from antipsychotic users (n=16 men and n=15 women) and age- and sex-matched non-users (n=80 men and n=75 women) participating in the Geelong Osteoporosis Study. Bone quality was determined using quantitative ultrasound (QUS) of the left calcaneus and included: Broadband Ultrasound Attenuation (BUA), Speed of Sound (SOS) and Stiffness Index (SI). Medication use and lifestyle factors were self-reported, anthropometry measured, and socio-economic status (SES) determined. Linear regression analyses were used to test cross-sectional associations between bone quality and antipsychotic use, after adjusting for potential confounders.

Results: Antipsychotic users were more likely to smoke and use antidepressants, were less active and consumed less alcohol. Otherwise, the groups were similar in regards to height, weight, SES and use of hormone therapy and bone active medications and supplementations. After adjusting for weight, antipsychotic use was associated with a 6.0% difference in mean BUA [109.8 (103.9-115.6) vs. 116.8 (114.2-119.4) dB/MHz, p=0.03] and 7.0% difference in mean SI [91.6 (84.8-98.4) vs. 98.5 (95.5- 101.5) %, p=0.07] compared to non-users. Associations persisted following further adjustment for mobility, alcohol consumption, smoking, SES and medications known to affect bone. There was no difference in SOS between antipsychotic users and non-users (p=0.29).

Conclusion: Use of antipsychotics was associated with lower QUS values. Hence, the risk of osteoporosis should be considered when antipsychotics are prescribed.

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Precision medicine in the bone clinic – its time is now.

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Case Description

The proband was referred to paediatric endocrinology at age 4 years with recurrent low trauma fractures including a left humeral spiral fracture at age 14 months, right wrist fracture at age 3 years, and recurrent metatarsal fractures aged 4 years. Bone densitometry demonstrated z scores of -1.8 (TBLH), -1.7 (AP spine), -3.3 (left total femur), and -3.1 (right total femur).

Her maternal uncle, grandfather and grandmother had severe osteoporosis on bone density and multiple low trauma fractures. Her mother had osteopaenia without fracture. A heritable bone fragility disorder was suspected.

The proband underwent panel sequencing for brittle bone disorders which identified a *PLS3* variant (heterozygous c.653A>G p.(Asp218Gly)) not previously reported in clinical or population databases, considered a variant of uncertain clinical significance (VUS). *PLS3* mutations are associated with X-linked osteogenesis imperfecta (1, 2).

A collaborative effort between treating endocrinologists facilitated cascade testing (via Sanger sequencing) which demonstrated appropriate segregation of the *PLS3* variant with phenotype, supporting this variant as likely pathogenic.

Discussion

Identifying monogenic causes of bone fragility benefits both patient and clinician through a better understanding of aetiology, prognosis, treatment approaches, and genetic counselling.

In the absence of an identifiable secondary cause, clinicians should have a low threshold for performing genetic testing in individuals with recurrent fragility fractures (particularly if presenting at young age or in men), those with extra-skeletal manifestations, and individuals with a suggestive family history. A lack of family history, however, should not dissuade genetic testing given up to two-thirds of COL1A1 and one-third of COL1A2 mutations occur de novo (3).

The time to incorporate genetic testing as part of routine care in the bone clinic is now. However, for this to occur, public laboratories need to be adequately resourced to facilitate timely turnaround, and ensure rebates are available for equitable access.

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Asia Pacific Fragility Fracture Alliance – Fragility Fracture Network Hip Fracture Registry Toolbox: A Resource to Support Registry Implementation

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Introduction: Hip fracture registries provide a mechanism to benchmark care provided by hospitals against clinical standards. As of May 2021, registries have been established in approximately one tenth of countries worldwide. A hip fracture registry toolbox is intended to provide practical tools to support registry development.

Method: The toolbox has been developed as a collaboration between the Asia Pacific Fragility Fracture Alliance (APFFA) Hip Fracture Registry Working Group and the Fragility Fracture Network (FFN) Hip Fracture Audit Special Interest Group.

Results: The toolbox summarises essential components of national quality improvement programmes for hip fracture care. This features best practice clinical standards, including quality indicators (e.g. measures relating to pain assessment, time to surgery, early mobilisation, secondary fracture prevention and multidisciplinary management). Hip fracture registries provide the technical infrastructure for hospital teams to benchmark the care they provide against quality indicators. The toolbox also focuses on practical aspects of registry establishment including clinical leadership and engagement, getting buy-in from diverse stakeholders, building the case for change, registry planning and funding, piloting a registry, governance and ethics considerations, and a minimum common data set and data dictionary.

A summary of the extensive literature on multidisciplinary care of hip fracture patients is provided, in addition to detailed case studies of national registries in Australia and New Zealand, Spain and the United Kingdom. A series of short interviews published on YouTube complement the toolbox with experience from leaders of well-established registries.

Conclusions: The APPFA-FFN hip fracture registry toolbox provides a distillation of the global experience to date in establishing national registries. The toolbox is free for download from www.apfracturealliance.org/HFR-toolbox/ and is intended to support colleagues throughout the world who would like to establish a registry in their country.

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Asia Pacific Fragility Fracture Alliance Primary Care Toolkit: Empowering Fragility Fracture Education

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Objective: The Asia Pacific Fragility Fracture Alliance (APFFA) is a federation committed to reducing the burden of low trauma fracture throughout the region. Education on fracture prevention to those at the forefront of patient care is an important part of this effort.

Method: APFFA has curated educational materials developed by others (https://apfracturealliance.org/education-directory/) and developed a Primary Care Physician (PCP) Education Toolkit (https://apfracturealliance.org/education-toolkit/). Here we describe the toolkit and report its introduction during the COVID-19 pandemic.

Results: The PCP Education Toolkit is designed as a half-day educational program together with supporting resources to highlight the role of primary care providers in this effort. The educational program includes a lecture focused on the burden of fracture, a lecture focused on clinical assessment of fracture risk, a discussion kit, and materials to assist with meeting planning. The discussion kit is designed to be adaptable to local practices and constraints. The supporting material features a patient handbook that gives practical advice on nutrition, home safety, and issues to be raised during medical encounters. COVID-19 hampered rollout of these materials.

In addition, APFFA has relied on its constituent organizations to provide educational content to promote best practices in acute fracture management, rehabilitation, and secondary fracture prevention through the development of an education directory. The directory includes synopses and links to high quality materials from around the world.

Conclusions: The PCP Education Toolkit was designed with the expectation that the program would be presented as live meetings. The pandemic made this infeasible. Despite the restrictions, the PCP Education Toolkit materials have been enthusiastically received in New Zealand and disseminated by Osteoporosis NZ. As the world emerges from the pandemic, we are looking to present this material in more venues in 2022 and beyond. The toolkit is available free of charge at the above address.

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Multimorbidity increases risk of osteoporosis under-diagnosis and under-treatment in patients at high fracture risk: 45 and Up a prospective population based-study

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Multimorbidity adds to clinical management complexity but its contribution to the osteoporosis treatment gap is unknown. This study aimed to determine the impact of multimorbidity on fracture risk and on osteoporosis management in high fracture risk persons.

45 and Up is a prospective population-based cohort of 267,153 people with questionnaires linked to hospital (Admitted Patients Data Collection –APDC), emergency (Emergency Department Data Collection – EDDC)[1], Pharmaceutical Benefits Scheme (PBS) and Medicare Benefits Schedule (MBS)[2] datasets. Fractures and Charlson Comorbidity Index (CCI) were identified from APDC and EDDC, DXA investigation from MBS; osteoporosis treatment from PBS.

There were 25,280 persons with incident fracture classified in high and low risk based on 10-year Garvan fracture risk (age, sex, weight, prior fracture and falls) threshold ≥20%.

Association of CCI with fracture risk was assessed by Cox model and likelihood of investigation and treatment initiation by logistic regression.

Persons in the high risk group were significantly older and had higher CCI than those in the low risk group. Having a higher CCI and being in the high risk group were independently associated with > 2-fold risk of re-fracture. However, in the high risk group, only 28% (48% women and 17% men) were investigated and 21% (24% women and 14% men) treated. A higher CCI was associated with significantly lower probability of investigation [OR, women: 0.84 (0.79-0.89); men: 0.71 (0.62-0.81)] and treatment initiation [OR, women: 0.87 (0.82-0.94); men: 0.75 (0.66-0.85)].

Multimorbidity was associated with higher fracture risk but lower likelihood of investigation or treatment for osteoporosis. These findings suggest that fracture risk is either under-estimated or under-prioritized in the context of multimorbidity, and highlights the need for improved fracture care in this setting.

[1] Data was linked by the Centre for Health Record Linkage

[2] MBS and PBS data sets were provided by Services Australia

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Opportunistic Screening for osteoporosis using L1 vertebral density on abdominal CT in an Australian population

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Background:

Osteoporosis is under-diagnosed in Australia, with 75% of subjects at risk of fragility fractures are not investigated or treated (1). Opportunistic osteoporosis screening in patients undergoing computed tomography (CT) scans can potentially avoid additional imaging and optimise patient convenience (2). We aim to assess the diagnostic accuracy of L1 vertebral HU measurement on abdominal CT scans with L1 DEXA T-score as the reference standard.

Methods:

Consecutive patients who underwent abdominal CT were included if they underwent DEXA scans within a 12-month period. Density values (HU) from CT at L1 were measured in the axial plane over a central 2 cm area of L1 trabecular bone. Pearson's correlation coefficient was used to quantify the correlation between L1 density and T-score. T scores were analysed categorically as per WHO definitions using different HU thresholds.

Results:

The study comprised 460 patients (42.8% males, 57.2% females). The prevalence of osteoporosis was 11.5%. CT density correlated with DEXA T-score (r=0.372, p<0.001).The AUC across CT density thresholds at L1 to distinguish osteoporosis from osteopenia and normal BMD was 0.65 for the entire cohort. In females, using a threshold of 190 HU detected T-scores ≤ -2.5 with a NPV of 94.8%, O.R. = 4.5, p < 0.01) and T-scores ≤ -1.5 with a NPV of 80.5%, O.R. = 4.1, p < 0.01). In males, a threshold of 180 HU detected T-scores ≤ -2.5 with a NPV of 100%, O.R. = 10.1, p < 0.01) and T-scores ≤ -1.5 with a NPV of 86.8%, O.R. = 2.6, p = 0.03).

Conclusion:

L1 HU values less than 190 and 180 increased the odds of osteoporosis diagnosis in an Australian female and male cohort. The use of abdominal CT to screen for osteoporosis is feasible and can improve the diagnosis rate of osteoporosis leading to reduced fracture risk.

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Automated best practice alerts improved treatment initiation post hip fracture

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Background:

An initial fragility fracture increases risk of subsequent fracture two- to three-fold; the highest risk is evident within the first two years (1). Despite the known benefit in prompt treatment post-fracture, underutilisation of anti-resorptive medications is widespread (2). The Australian & New Zealand Hip Fracture Registry shows hip fractures, the fracture with the highest morbidity and mortality, remain sub optimally managed (3, 4). After consultation with stakeholders, Best Practice Alerts (BPA) were implemented with a built-in treatment pathway to improve Vitamin D testing, inpatient Vitamin D treatment, and pre-discharge anti-resorptive treatment initiation in patients with hip fracture.

Methods:

Hip fracture admission pre-BPA implementation was captured via the REStORing health of acutely unwell adulTs cohort (May 2019 – March 2020), and via electronic medical record post-BPA implementation (March - July 2021). Three BPAs were implemented: 1) order for Vitamin D testing triggered by inpatient hip fracture diagnosis, 2) order for colecalciferol triggered by vitamin D result ≤50 nmol/L AND vitamin D not already charted, 3) order for anti-resorptive treatment triggered by the discharge summary. The introduction of BPAs was supported by targeted education of stakeholders. Patient discharge medications were compared pre- and post-BPA implementation.

Results:

BPA fired 572 times in 75 hip fracture patients [age (mean ± SD) 79.5±8.9yrs, 61.3% female]. Parameters which did not differ pre- (n=58) and post-implementation were vitamin D testing (96.6% vs 97.3%), vitamin D level (62.5 vs 68.3 nmol/L), vitamin D treatment at discharge (75.9% vs 88%) and anti-resorptive treatment on admission (15.5% vs 20%). Anti-resorptive treatment rate on discharge increased 3-fold post-BPA implementation (21% vs 68%, p=<0.001).

Conclusion:

Automated BPA with an incorporated evidence-based treatment pathway provides a powerful tool to assist medical staff in overcoming the secondary fracture prevention care gap. Further fine-tuning will reduce redundant firing of BPA and avoid “alert fatigue”.

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Bone matrix quality in transiliac biopsies from post-menopausal osteoporotic (PMOP) women treated with denosumab (DMab) for up to 10 years (FREEDOM and FREEDOM Extension Trials)

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In PMOP women, DMAb therapy through 10 years (y) is associated with continued BMD gains and low fracture incidence. Until 5y, DMAb significantly increased the degree of mineralisation of bone (DMB) compared with placebo (Pbo), then plateaued through 10y in both cortical (Ct) and cancellous (Cc) bone. We assess the microhardness (Hv) of bone reflecting elastic and

plastic deformations by DMB and the quality of mineral and organic matrix in bone biopsies from PMOP women treated with DMAb for up to 10y.

Assessments were performed blindly on 1) 72 iliac bone samples from patients treated for 2/3 y either Pbo or DMAb, and 2) 49 iliac bone samples from patients treated for 5y (N=28) or 10y (N=21) with DMAb.

After 2/3y, Ct Hv was significantly higher in DMAb than Pbo (Table) with increased mineral/matrix ratio but without significant variation of other quality of mineral and organic matrix variables. At 5y and 10y, mineral/matrix ratio, mineral maturity (transformation of precursors into apatite crystals), crystallinity (size/perfection of crystals), were higher compared to 2/3y of DMAb, supporting transition to more mature crystals (within physiological range). However, Hv was significantly lower at 5y and 10y than to 2/3y while Cc collagen maturity was increased.

In conclusion, DMAb improves bone Hv after 2/3y, mainly by changes in bone matrix mineralisation characteristics that are consistent with DMAb's mechanism of action as a potent remodelling inhibitor. However, persistently low state of bone remodelling at 5y and 10y is associated with reduced bone Hv, suggesting aging of the poorly remodelled organic matrix (non-collagenous proteins or other factors could be involved). At the organ level, DMAb preserves modelling-based bone formation at weight-bearing sites and leads to continued BMD gains and low fracture rate up to 10y.

Table

Treatment / Variables	Placebo 2-3 years	Denosumab 2-3 years	Denosumab 5 years	Denosumab 10 years
Cortical bone (Ct)				
Microhardness (Hv)	53.56 ± 3.07	**56.28 ± 3.11** [a]	**50.18 ± 3.86** [b]	**50.38 ± 3.41** [c]
Mineral/matrix	4.43 ± 0.29	**4.65 ± 0.26** [a]	**4.85 ± 0.28** [b]	**4.91 ± 0.37** [c]
Mineral maturity	1.88 ± 0.26	1.88 ± 0.26	**2.13 ± 0.45** [b]	**2.46 ± 0.64** [c]
Mineral Crystallinity	0.0373 ± 0.0016	0.0381 ± 0.0015	**0.0394 ± 0.0014** [b]	**0.0399 ± 0.0009** [c]
Mineral Carbonation	0.0073 ± 0.0004	0.0073 ± 0.0005	**0.0068 ± 0.0004** [b]	**0.0068 ± 0.0004** [c]
Collagen maturity	4.48 ± 0.47	4.42 ± 0.45	4.60 ± 0.33	4.45 ± 0.68
Cancellous bone (Cc)				
Microhardness (Hv)	56.53 ± 4.54	56.26 ± 3.72	**53.14 ± 2.60** [b]	**52.82 ± 3.15** [c]
Mineral/matrix	4.39 ± 0.35	**4.62 ± 0.35** [a]	**4.95 ± 0.23** [b]	**4.98 ± 0.17** [c]
Mineral maturity	1.54 ± 0.25	1.53 ± 0.24	**1.80 ± 0.29** [b]	**2.05 ± 0.37** [cd]
Mineral Crystallinity	0.0373 ± 0.0021	0.0379 ± 0.0021	**0.0397 ± 0.0015** [b]	**0.0404 ± 0.0012** [c]
Mineral Carbonation	0.0075 ± 0.0005	0.0073 ± 0.0006	**0.0067 ± 0.0014** [b]	**0.0068 ± 0.0005** [c]
Collagen maturity	3.98 ± 0.68	3.86 ± 0.72	**4.31 ± 0.52** [b]	**4.46 ± 0.34** [c]

Bone matrix quality variables are summarised with mean ± SD. Intrinsic quality of the mineral and organic matrix were assessed using Fourier transform infrared microspectroscopy. Hv was measured with a Vickers microindenter. Data were obtained separately in Ct and Cc. P-values were calculated using Wilcoxon rank sum test for pairwise comparison.

a : $p<0.02$ Denosumab Year 2/3 vs Placebo Year 2/3

b : $p\leq0.008$ Denosumab Year 5 vs Denosumab Year 2/3

c : $p\leq0.006$ Denosumab Year 10 vs Denosumab Year 2/3

d : $p<0.02$ Denosumab Year 10 vs Denosumab Year 5

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Changing anti-resorptive prescription rates in the last five years

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Background

Osteoporosis or osteopaenia affect approximately six million Australians aged > 50 years, resulting in fracture-related morbidity and mortality. The treatment dosing schedule, duration and effects after treatment cessation vary due to the different actions of the anti-resorptive medications. Importantly, a rapid increase in bone turnover markers and reduction in bone mineral density can be seen following denosumab cessation (1). Vertebral fractures have been observed eight months following the last denosumab dose due to the rebound increase of bone resorption, in contrast to bisphosphonates' persistent skeletal action despite cessation (2). Lockdown of citizen movement in Australia occurred during the COVID-19 pandemic in 2020 causing disruption to healthcare and in-person reviews.

Aim

To examine national prescribing rates from 2016 to 2021 of denosumab, alendronate and risedronate.

Method

This retrospective audit analysed prescribing rates of anti-resorptive medications. Data was sourced from The Pharmaceutical Benefits Scheme 'Date of Supply'. Time-based trends were analysed by two methods: a polynomial (quadratic) line of best fit (R^2 = 0.8639) and an interrupted time series using a quasipoisson distribution, with comparison made between pre- and post-COVID-19 onset (March 2020) periods.

Results

Prescription rate of denosumab increased from 2016 to 2021. The rate has been steadily slowing with intensification of this trend noted post the onset of the COVID-19 pandemic (Figure 1). The long-term rates of prescription of alendronate and risedronate have decreased, with a notable inversion in this trend following March 2020 (Figure 2).

Conclusion

The rate of denosumab prescriptions has slowed, more so following March 2020. This could be related to decreased new starts and/or decreased treatment continuation. Future research is required to determine if higher rates of rebound-associated fractures are occurring. Clinicians are urged to ensure that a strict 6 monthly dosing interval for denosumab is employed to mitigate the risk of rebound fractures.

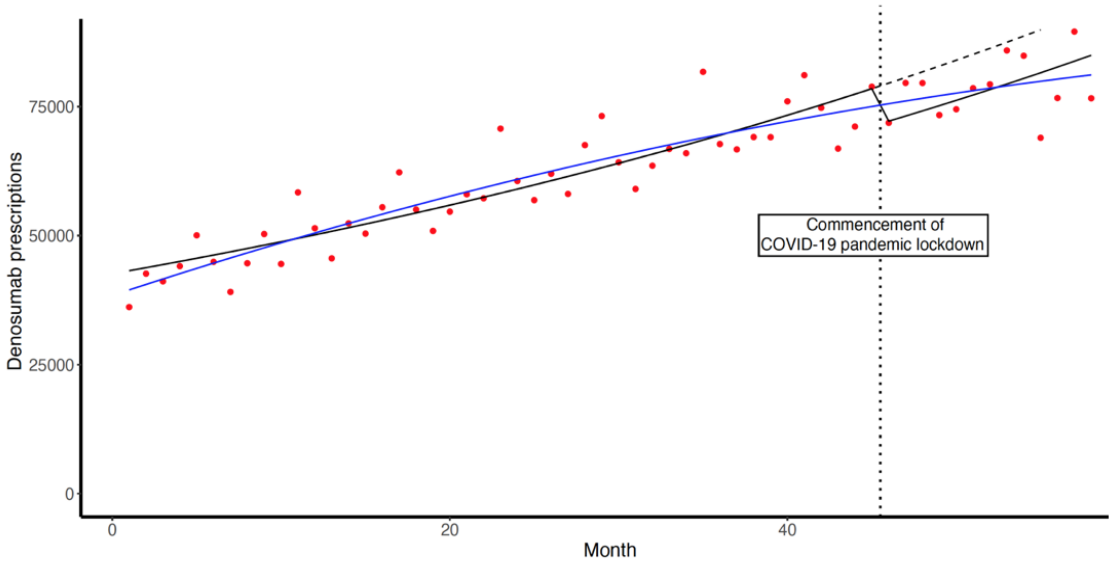

Figure 1. Prescription rates of denosumab 2016 to 2021. Dots=number of denosumab prescriptions per calendar month; black line=quasipoisson generalised linear model of best fit; blue line=predicted line of best fit assuming no interruption (R^2=0.8639).

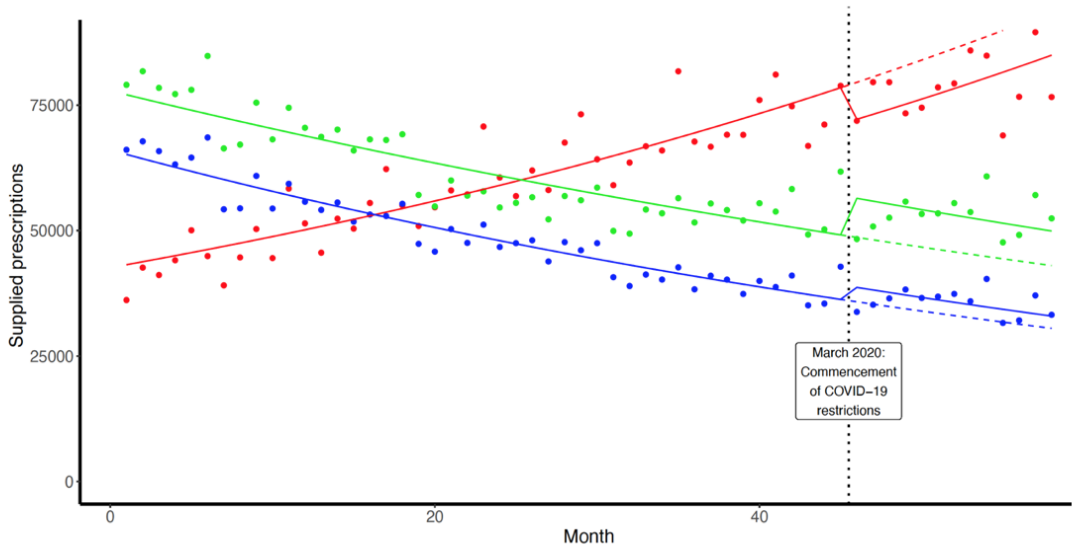

Figure 2. Prescription rates of anti-resorptive medications in Australia 2016 to 2021. Dots=number of prescriptions per calendar month. Red line=quasipoisson generalised linear model of best fit for denosumab prescription rates; green line=quasipoisson generalised linear model of best fit for risedronate prescription rates; blue line=quasipoisson generalised linear model of best fit for alendronate prescription rates.

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Association of abdominal aortic calcification with peripheral quantitative computed tomography bone measures in older women: The Perth Longitudinal Study of Ageing Women

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Background: Abdominal aortic calcification (AAC), a marker of advanced atherosclerotic disease, is associated with two-dimensional areal bone density. However, limited studies have assessed its association with three-dimensional pQCT-derived bone outcomes. This is important to further understand the relationship between vascular and bone health, considering the importance of such outcomes to whole-bone strength and fracture resistance. This study examined associations of abdominal aortic calcification (AAC) with total, cortical and trabecular volumetric bone density (vBMD), bone structure and strength among community-dwelling older women.

Methods: A sub-sample of women (n=648; mean±SD age 79.7±2.5 years) from the Perth Longitudinal Study of Aging in Women (PLSAW) were included with AAC assessed on lateral DXA images at 1998/1999 and 2003. We assessed associations between cross-sectional (in 2003) and longitudinal (progression from 1998/1999-2003) AAC with cross-sectional (in 2003) and longitudinal (change from 2003-2005) pQCT measures. Bone density, structure and strength outcomes were derived from pQCT scans at the 4% radius and tibia (predominantly trabecular), and 15% radius (predominantly cortical).

Results: Partial (adjusted for age, BMI, calcium treatment) Spearman correlations revealed no cross-sectional associations between AAC and any pQCT bone measures in 2003. AAC progression was inversely associated with 4% radius total bone area (r_s= −0.088, p=0.044) in 2003, with trends for similar associations with 4% tibia and 15% radius total bone area (both p<0.06). Neither AAC in 2003 nor AAC progression were associated with subsequent 2-year pQCT bone changes. ANCOVA showed no consistent differences in bone outcomes between women with and without AAC, with and without AAC progression, nor across categories of AAC extent.

Conclusion: In older women, AAC was not consistently associated with vBMD, bone structure or estimated bone strength at the tibia or radius, nor with longitudinal changes in these outcomes. Further research is required to better understand the nexus between the vasculature and bone.

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PREVALENCE AND ASSOCIATES OF AGE-RELATED MUSCLE STRENGTH DECLINE IN COMMUNITY DWELLING OLDER ADULTS – COMPARISONS OF EAST AND WEST

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Background

Age-related decline in muscle strength is associated with adverse clinical outcomes including fragility fracture. However, no previous study has compared its prevalence, and demographic and lifestyle associates, in comparable populations in different geographic regions. We considered this here.

Methods

We used 2 community based cohorts; UK study participants (1572 men; 1415 women) were recruited from the Hertfordshire Cohort Study, while Japanese participants (520 men; 1028 women) were recruited from the ROAD study. Lifestyle questionnaire data from the two cohorts were harmonised. Age-related muscle strength decline was measured in both studies using a dynamometer, with low values classified as grip strength of <30kg in men and <20kg in women.

Results

The median age of UK participants was 65.8 (IQR 63.5 – 67.8) years in men and 66.5 (IQR 64.5-68.7) years in women, while in Japan this was 68 (IQR 58-76) years in men and 67 (57-74) years in women. The prevalence of age-related muscle strength decline in the UK was 3.0% in men and 10.3% in women, lower than Japan (11.5% men and 16.1% women). In both cohorts, women were at greater risk of muscle strength decline (UK: OR 3.73, 95% CI 2.66,5.23, p<0.001; Japan: OR 1.47, 95% CI 1.07-2.01, p=0.02), while greater height was protective (p<0.001). Smoking was not associated with muscle strength decline in either cohort, though drinking alcohol was protective in Japanese women (OR 0.60, 95% CI 0.38-0.95, p=0.03). Age at leaving education was a predictor of age-related muscle strength decline in both cohorts, particularly in Japan, where more time spent in education was protective in both genders (p<0.001); in the UK, this was significant in women only (p=0.01).

Conclusions

Despite a different prevalence of age-related muscle strength decline in comparable cohorts in UK and Japan, the anthropometric and lifestyle determinants of the condition were very similar.

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Higher levels of circulating osteoprogenitor cells are associated with higher bone mineral density and lean mass in older adults, and are a putative biomarker for osteoporosis: A cross-sectional study.

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Circulating osteogenic precursor (COP) cells are a heterogeneous population of cells that circulate within the peripheral blood with characteristics of the bone marrow mesenchymal stem and progenitor pool. Little is known about the behavior of this cell population in humans. The aim of this study was to identify whether a relationship exists between COP cells (as a percentage of the peripheral blood monocytic cells) and musculoskeletal morphometry and to identify if COP have potential clinical utility as a biomarker for osteoporosis. We recruited 57 older adults (Median age: 69, IQR: 65, 75) living independently in the community and performed cross-sectional analysis to identify associations between the percentage of COP cells and body composition parameters, and through receiver operating characteristic analysis, evaluated their ability to act as a biomarker of osteoporosis. COP cells were moderately associated with whole-body bone mineral density (BMD) (r=0.323, p=0.014) and bone mineral content (BMC) (r=0.387, p=0.003), neck of femur BMD (r=0.473, p <0.001) and BMC (0.461, p <0.001) as well as appendicular lean mass (ALM) (p=0.038) and male sex (p=0.044) in univariable analysis. In multivariable analysis controlling for age, gender, height and weight, COP cells remained strongly associated with neck of femur BMD (p=0.001) and content (p=0.003). COP cells were also a good predictor of osteoporosis (DXA T-score <-2.5) at the neck of femur (cutoff: 0.4%, sensitivity: 100%, specificity 79%) and total body (cutoff: 0.35%, sensitivity: 80%, specificity: 81%). This study shows strong relationships between bone parameters and COP cell number and ALM and male sex. They also have potential as a biomarker of osteoporosis, which may provide a new tool for advanced detection and screening in clinical settings. Future larger evaluation studies should verify the cut-offs for biomarker use, and further explore the relationship between COP cells and muscle.

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OsteoPreP: The effect of probiotic supplementation on bone, muscle, and glucose metabolism in postmenopausal women – study design

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Osteoporosis is a disease characterized by loss of bone mineral density (BMD) and deteriorated bone microstructure, resulting in reduced bone strength and increased risk of fracture. Loss of BMD occurs from the third decade of life, but women experience an accelerated bone loss following menopause. We have previously shown that daily supplementation with the probiotic *Lactobacillus reuteri* for 12 months reduces bone loss in older women with low bone mass(1), but the underlying mechanism remains to be elucidated. Recent studies in mice have demonstrated that short-chain fatty acids (SCFAs) produced by the gut microbiota regulate bone mass via induction of regulatory T cells resulting in increased bone formation and decreased bone resorption. The primary aim of the OsteoPreP trial is to investigate if daily consumption of a probiotic supplement containing inulin (a prebiotic soluble fibre) twice daily for 12 months can prevent the rapid bone loss in early postmenopausal women. Secondary aims will focus on intestinal SCFA levels, gut microbiota composition, immune system modulation as well as musculoskeletal and metabolic function as potential mediators.

One hundred and sixty postmenopausal women (aged 40-65 years, 1 to 4 years since final menses) will be recruited from the community to participate in this single-site, double-blind, placebo-controlled trial. Participants will be randomized to receive the active supplement or placebo. The percentage change from baseline in BMD and bone microarchitecture at 12 months will be measured with high-resolution peripheral quantitative computed tomography. Secondary endpoints include stool SCFA levels, gut microbiome analysis by shotgun metagenomics, blood regulatory T cell numbers, blood glucose and glycaemic variability, cognition, ambulatory blood pressure, and muscle tissue function.

If long-term consumption of this probiotic supplement can protect against bone loss in postmenopausal women, this intervention can be used to effectively prevent osteoporosis and potentially associated musculoskeletal and metabolic conditions.

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Development of a novel method to measure bone marrow fat fraction in older women using high-resolution peripheral quantitative computed tomography

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Marrow adipose tissue (MAT) has been implicated in a number of conditions associated with bone deterioration and osteoporosis. Several studies have found an inverse relationship between MAT and bone mineral density (BMD), and higher levels of MAT in those with prevalent fracture. Magnetic resonance imaging (MRI) is the gold standard for measuring MAT but its use is limited by high costs and low availability. We hypothesized that MAT could also be accurately quantified using high-resolution peripheral quantitative computed tomography (HR-pQCT).

In the present study, a novel method to quantify the tibia bone marrow fat fraction, defined by MRI, using HR-pQCT was developed. In total, 38 postmenopausal women (mean [standard deviation] age 75.9 [3.1] years) were included and measured at the same site at the distal (n=38) and ultradistal (n=18) tibia using both MRI and HR-pQCT. To adjust for partial volume effects, the HR-pQCT images underwent 0 to 10 layers of voxel peeling to remove voxels adjacent to the bone. Linear regression equations were then tested for different degrees of voxel peeling, using the MRI-derived fat fractions as the dependent variable and the HR-pQCT-derived radiodensity as the independent variables.

The most optimal HR-pQCT-derived model, which applied a minimum of 4 layers of peeled voxel and with more than 1% remaining marrow volume, was able to explain 76% of the variation in the ultradistal tibia bone marrow fat fraction, measured with MRI ($p<0.001$).

The novel HR-pQCT method, developed to estimate MAT, was able to explain a substantial part of the variation in the bone marrow fat fraction and can be used in future studies investigating the role of MAT in osteoporosis and fracture risk prediction.

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Sex differences between bone health, and obesity, sarcopenia, and sarcopenic obesity in Indian older adults.

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Background: Sarcopenia and obesity influence bone health however, there is limited data on these associations in ethnic populations where there are differences in body composition. This study investigated sex differences between BMD and bone mineral apparent density (BMAD) and obesity, sarcopenia and sarcopenic obesity among Indian older adults.

Methods: 1057 adults aged ≥50 years were included. Dual-energy X-ray absorptiometry measured BMD at the hip, spine and whole-body; and body composition (lean and fat mass) and hand grip strength was assessed. Obesity was defined by body fat percentage (>25% for men and >35% for women), and sarcopenia was defined using the revised Asian Working Group for Sarcopenia classification. BMAD was calculated as BMD/√bone area. Participants were classified into four groups: non-sarcopenic non-obesity (NSNO), obesity (O), sarcopenia (S) or sarcopenic obesity (SO). Linear regression (β-coefficients and 95%CI) was performed with adjustments for age, sex, smoking status, education and occupation type.

Results: Prevalence of S (37%) and SO (6%) were higher in men than women (17% and 4%, respectively). Older men with O had lower whole-body BMD (-0.055; -0.078, -0.032) and BMAD (-0.002; -0.002, -0.001) than NSNO, but men with SO had lower hip, spine and whole-body BMD and BMAD (all $p<0.05$) than NSNO. Older women with S had lower hip and spine BMD and BMAD than NSNO, but those with obesity had higher hip and spine BMD and BMAD (all $p<0.05$) than NSNO.

Conclusion: In Indian older adults, obesity is associated with higher bone density, while sarcopenia is associated with lower bone density in women. Sarcopenic obesity is associated with poorer bone density at all three sites in men. Future studies should focus on understanding the contributions of body composition in ethnic populations to poor bone health to develop targeted effective interventions for sarcopenia, obesity, and osteoporosis in Indian older adults.

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Asymptomatic cerebrovascular disease, falls and fracture risk in older Australian women: The Perth Longitudinal Study of Ageing Women

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Publish consent withheld

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Crystal bone is a personalised short-term fracture risk prediction with natural language processing methods

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Common fracture risk assessment tools e.g. FRAX and Garvan, confer long-term but not short-term risk estimates necessary to identify patients likely to fracture in the next 1–2 years. Furthermore, these tools utilise cross-sectional data representing a subset of all available clinical risk factors for risk prediction. Thus, these methods are generalised across patient populations and may not fully utilise patient histories in electronic health records (EHRs) that contain temporal information for thousands of unique features.

We used the Optum® EHR dataset to develop Crystal Bone, a method that applies machine learning techniques to predict fracture risk over a 1–2 year timeframe. Crystal Bone uses context-based embedding techniques to learn an equivalent “semantic” meaning of various medical events. Similar to how language models predict the next word in a given sentence, Crystal Bone can predict that a patient’s future trajectory may contain a fracture or that the “signature” of the patient’s overall journey is similar to that of a typical fracture patient.

We applied Crystal Bone to two datasets, one enriched for fracture patients and one representative of a typical hospital system. When predicting likelihood of fracture in the next 1–2 years, Crystal Bone had an area under the receiver operating characteristic (AUROC) score ranging from 72%–83% on a test (hold-out) dataset. These results suggest performance similar to FRAX and Garvan, which have 10-year fracture risk prediction AUROC scores of 64.4% +/– 3.7%.

In conclusion, it is possible to use each patient’s unique medical history as it changes over time to predict patients at risk for fracture in 1–2 years. Furthermore, it is theoretically possible to integrate a model like Crystal Bone directly into an EHR system, enabling “hands-off” fracture risk prediction, which could lead to improved identification of patients at very high risk for fracture.

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Randomised study to evaluate a secondary prevention program for women with osteoporotic fractures

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We assessed the impact of an intensive outreach program in postmenopausal women (age 67–85y) with osteoporosis over 3, 6, and 12 months (mos). Medicare Advantage members with evidence of a fragility fracture (fx) from 15Apr2016–27Jan2017 and 23Oct2017–27Nov2017 were eligible. Randomisation was to a current health plan program (standard-of-care [SOC]); or an intensive outreach intervention, which, in addition to SOC, included educational mailing to patients, post–heel scan follow-up phone calls to patients and providers, and an informational fax and phone call to providers. Study endpoints were: 1) composite of DXA monitoring and/or osteoporosis medication fill, 2) DXA monitoring, 3) osteoporosis medication fill, and 4) subsequent fxs.

Over 12 mos, 3,720 patients (1,847 intervention; 1,873 SOC) were followed up; only 98 (5.3%) patients and 678 (36.7%) providers were eligible for phone outreach and successfully contacted. The results were consistent across mos 3, 6, and 12. At 12 mos, the intervention group had a statistically significant greater proportion of DXA monitoring and/or osteoporosis medication fill vs.

SOC (32.7% vs. 29.5%; p=0.036) and a greater proportion of DXA monitoring alone vs. SOC (29.9% vs. 26.1%; p=0.009). No significant differences between intervention and SOC were found for medication fill alone (10.5% vs. 10.1%, respectively; p=0.759) or subsequent fxs (21.0% vs. 21.7%; p=0.591). The overall osteoporosis medication fill for all patients was 6.8% within 6 mos and 10.3% within 12 mos. Further, 21.4% of all patients experienced an additional fx, and 4.4% experienced multiple fxs.

In conclusion, the intensive outreach program increased post-fx DXA monitoring vs. SOC, with no differences between groups in medication fill or subsequent fxs. Addressing the barriers encountered in this study to increase contact with patients and providers may be needed to increase osteoporosis monitoring and use of osteoporosis medication and reduce subsequent fxs during post-fx care.

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Probability of achieving T-scores goals above -2.5 with alendronate (ALN) or romosozumab (ROMO) followed by alendronate or denosumab (DMAB)

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Background: Increases in bone mineral density (BMD) reduce fracture risk in patients receiving treatment for osteoporosis. Goal-directed Treatment (also called 'Treat-to-Target') recommends that selection of initial treatment (anti-resorptive agent or bone-forming agent) for patients with a T-score <−2.5 should be based on the probability of achieving a goal BMD T-score ≥−2.5.

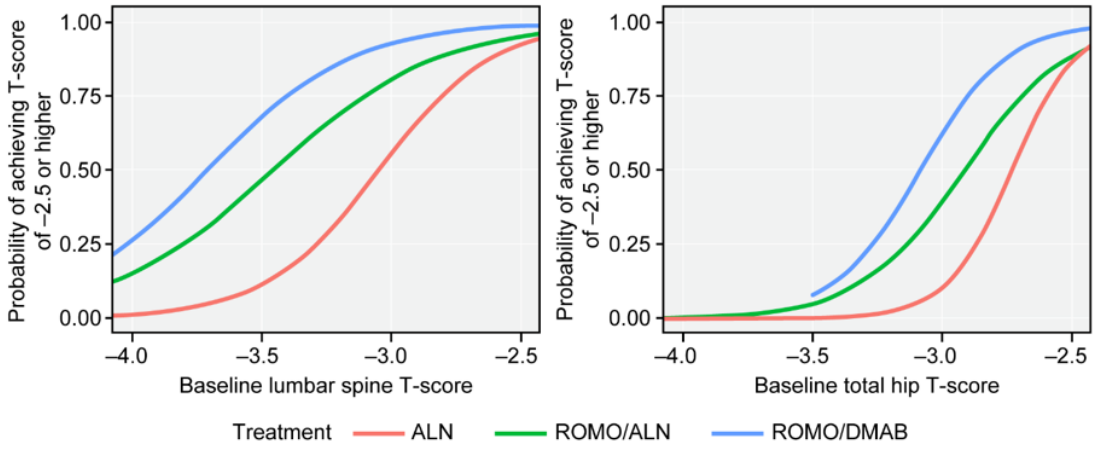
Objective: To compare the probability of achieving a T-score of ≥−2.5 at the total hip or lumbar spine after 3 years treatment with ALN only; or the treatment sequence of 12 months ROMO, followed by 2 years ALN (ROMO/ALN) or DMAB (ROMO/DMAB).

Methods: Female participants in the ARCH trial received ALN for 3 years or ROMO for 1 year followed by ALN for 2 years. Those in the FRAME trial received ROMO for 1 year followed by DMAB for 2 years. For participants with initial BMD T-scores <–2.5 at total hip or spine, we calculated the probability of achieving a T-score ≥–2.5 with the three treatments.

Results: The probabilities of achieving a T-score ≥–2.5 depended on baseline T-score and treatment; see Figure 1 for details.

Conclusion: Women with a baseline T-score ≥–3.0 at the spine or ≥–2.7 at the hip have at least a 50% chance to achieve a T-score ≥–2.5 with any of the three regimens. In contrast, those with a T-score below –3.0 at the spine, or –2.7 to –3.5 at the total hip, have a substantially greater probability of achieving T-scores ≥–2.5 when using a bone-forming agent first (i.e. ROMO/ALN or ROMO/DMAB vs. ALN alone). Those with hip T-scores ≤–3.5 may require more than 3 years of treatment that continues to improve BMD. These probabilities should be considered when selecting initial treatment.

Figure 1. Probability of achieving lumbar spine (left) and total hip (right) T-scores of –2.5 or higher at year 3. Participants with baseline spine T-scores of ≥–3.0 had a ≥55.3% probability with ALN, ≥80.7% with ROMO/ALN and ≥92.8% with ROMO/DMAB; those with a spine T-score of –3.5 had a 11.2% probability with ALN, 46.3% with ROMO/ALN, and 68.1% with ROMO/DMAB. The probabilities of reaching a T-score ≥–2.5 were lower for the total hip but trended similarly by treatment sequence; ≥50% of participants with an initial T-score of ≥–2.7 achieved a T-score ≥–2.5 with any of the treatments at both sites, those with a hip T-score of ≥–3.0 had a ≥10.2% probability with ALN, ≥39.2% with ROMO/ALN, and ≥62.1% with ROMO/DMAB; participants with a total hip T-score of –3.5 had a very low probability of reaching a T-score ≥–2.5 after 3 years of any treatment.



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Hip fracture has a significant contribution to fragility fracture burden in Ontario Canada

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We conducted a retrospective observational study to examine the contribution of hip fractures to fragility fracture burden in Ontario, Canada. Outcomes included: index and subsequent fracture type and date, patients requiring surgery, 30-day post-surgery complication rate, 1-year mortality rate and total healthcare cost per-patient in the 1st year after index fracture (IF).

115,776 patients were included; median age was 81 (IQR: 74–87) years and 72.3% were female. Hip fracture was the most common IF (27.3%, n=31,613), and 32.4% (n=10,254) of index hip fractures occurred in patients ≤80 years of age. Proportion of IF that were hip fractures by age was: 66-70, 12.1% (n=2,179); 71-75, 17.3% (n=3,092); 76-80, 24.2% (n=4,983); 81-86, 31.2% (n=7,524); and 86+, 39.3% (n=13,835). Hip fracture was also the most common 2nd fracture (27.8%, n=5,745); occurring as the 2nd fracture in ≥19% of cases for all IF sites examined, often after hip (33.0%) or pelvic (32.3%) IF, and least often after tibia/fibula/knee (23.3%) or radius/ulna (19.4%) IFs. Among patients requiring surgery related to their IF (n=44,949) and those experiencing complications 30 days post-surgery (n=8,868), respectively, 64.1% and 71.9% had a hip fracture. One-year all-cause mortality was 26.2% after hip IF and 15.9% in the entire cohort; hip fracture had the highest mortality rate of all IF sites examined, followed by femur (21.9%). Total mean (± SD) healthcare cost per-patient (in 2017 Canadian dollars) in the 1st year after IF was the 2nd highest for hip IF ($62,793 ± 44,438), with femur IF having the highest cost ($65,490 ± 54,116).

These data highlight the significant morbidity, mortality and financial burden of hip fragility fractures in adults aged >65 and the urgent need to initiate secondary fracture prevention measures after a fragility fracture occurring at any site to help reduce subsequent hip fracture and associated burden.

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Diagnostic test accuracy of self-reported use of medicines for bone health

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Background: Self-reported medicine use is often used in both research and clinical settings. This systematic review investigates the accuracy of self-reported medicine use compared to dispensed medicines for bone health.

Methods: Studies were included in the systematic review if the population of interest was community-dwelling or population-based samples of adults, taking at least one regular medicine for bone health ('Drugs for treatment of bone diseases' ATC code M05 and 'Mineral supplements' A11). Full text English-language papers published in peer reviewed journals were included where the full text paper was available. Studies were included if they compared self-reported medicines for bone health to dispensed medicines records. Dispensed medicines were sourced from either administrative claims data or from pharmacy dispensing records. Data were extracted and sensitivity and specificity, were calculated using Review Manager software version 5.4.1.

Results: The initial search returned 4465 studies, of which 147 full-text papers were retrieved for further consideration. Five studies met our selection criteria, representing a total of 8223 participants (5393 female). Three studies investigated 'Drugs for treatment of bone diseases', while the remaining studies investigated both 'Drugs for treatment of bone diseases' and 'Mineral supplements'. For 'Drugs for treatment of bone diseases' sensitivity ranged 0.54-0.80 while specificity ranged 0.97-1.00. We were unable to determine sensitivity for one study of pregnant women as no participants used bone related medicines. The highest sensitivity was demonstrated in a population of postmenopausal women, while the lowest sensitivity was demonstrated in an Australian population of adults aged over 45yr. For 'Mineral supplements' sensitivity ranged 0.67-0.71 while specificity ranged 0.92-1.

Discussion: Medicines for bone health demonstrated good specificity but only moderate sensitivity. This suggests self-report may be useful in identifying individuals who are not using medicines for bone health but may not capture all participants using medicines for bone health.

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The association between macrosomia and child bone density

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Introduction: Some evidence, but not all, has shown that higher birthweight is positively associated with bone density. Conversely, some evidence has shown that large for gestational age babies, or macrosomia, is associated with a reduced bone density (BMD) at birth. However, it is unknown if this pattern of association persists into childhood. Thus, we aimed to determine the association between macrosomia and bone mineral density in late childhood.

Methods: Women were recruited during pregnancy as part of the Vitamin D in Pregnancy Study at the University Hospital (formerly Geelong Hospital) antenatal clinic (n=475). At birth 400 mother-child pairs remained, 195 of whom returned at the 11-year follow-up and provided DXA measures (Lunar Prodigy). The regions of interest were Anterior-Posterior spine (L2-L4) and Total Body Less Head (TBLH). Macrosomia was defined as a birthweight equal to or greater than 4kg. Linear regression models were adjusted for maternal BMI during early pregnancy, child sex, and child pubertal stage. Models were then further adjusted for child's current size parameters (height and weight).

Results: There were 185 mother-child pairs with complete information for the current analyses. 36 (19.5%) children were above 4kg. There was a positive association between macrosomia and child spine BMD (β:0.04; 95%CI:0.01,0.08), BMC (β:3.44; 95%CI:1.19,5.69), and TBLH BMD (β:0.04; 95%CI:0.01,0.06) and BMC (β:167.90; 95%CI:66.01,269.80). After adjustment for child's current height and weight these associations were no longer significant.

Conclusion: In later childhood there was a positive association between macrosomia and bone mineral density. Thus, our data suggest that by age 11 years, there is an apparent positive association between macrosomia and BMD which is opposite direction to reports described at birth. This association appears to be largely driven by child's current size parameters, likely owing to increased mechanical loading in larger children.

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Maternal dysglycaemia in pregnancy and offspring bone health

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Introduction: Adults and children with diabetes mellitus have poorer bone health compared to healthy populations. Poorer bone health measured by ultrasound has been reported in infants born to mothers with gestational diabetes, however no study has

examined these associations beyond infancy. Thus, we aimed to determine the association between maternal dysglycaemia during pregnancy and offspring quantitative ultrasound (QUS) bone measures in childhood.

Methods: Singleton pregnancy women (n=475) at <16 weeks gestation were recruited from the University Hospital Geelong as part of the Vitamin D in Pregnancy Study. At 28-32 weeks gestation, 379 women underwent glucose challenge tests (GCT); results ≥8.00mmol/L were considered high. At the 11-year follow-up, 189 mother-child pairs returned, where children underwent QUS using an Achilles Insight Ultrasonometer at the left calcaneus, which measured bone speed of sound (SOS) (m/s), broadband ultrasound attenuation (BUA) (db/MHz) and stiffness index (SI). The average of two measurements of SOS, BUA and SI were used for analysis. Linear regression models were adjusted for child birthweight, child height, weight, sex and pubertal stage at 11 years, and maternal BMI and smoking status at recruitment. Of women who had a GCT, 164 children had QUS and adjustment measurements at 11 years.

Results: Twenty-four (14.6%) women had high GCT results. There was a weak positive trend for an association between a high GCT and child BUA (β: 3.80; 95%CI: -0.36,7.96; p=0.07) and SI (β: 3.87; 95%CI: -0.77,8.51; p=0.10), however this failed to reach significance. No association was observed between a high GCT and child SOS (β: 2.17; 95%CI: -7.84,12.18; p=0.67).

Conclusion: Maternal dysglycaemia during pregnancy was weakly associated with increased child bone measures at 11 years, however this study may be underpowered. This study lays the foundation for future larger studies and to investigate whether increased bone density is associated with a decreased fracture risk.

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X-linked Hypophosphataemic Rickets in Australian Children: Prevalence and Burden of Disease

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X-linked Hypophosphataemic Rickets in Australian Children: Prevalence and Burden of Disease

Aim

1. Estimate the national prevalence of x-linked hypophosphataemic rickets (XLH) in Australia.
2. Describe the prevalent cases in terms of:
3. Demographics
4. Family history of XLH
5. Presenting and current biochemical, clinical features and complications
6. Therapies used to treat XLH

Methods

Case definition, child <18 years with XLH: Rickets during childhood **AND** Pathogenic mutation in the PHEX gene **OR** iFGF23 levels above the limits of the local laboratory range **OR** Family history that supports X-linked inheritance.

The study was conducted with the Australian Paediatric Surveillance Unit (APSU). In June and July 2020, 1434 paediatricians were asked if they had seen a child with XLH. Paediatricians completed a case report form for each child into a REDCap database or sent to APSU via email.

Results:

47 paediatricians reported 1 or more cases of XLH. After exclusion of duplicates (10) and errors (5) there were 74 confirmed cases. The minimum national prevalence was 1.31 per 100,000 children under 18 years (CI 1.02 – 1.64). Current medial age 11.0 years (1-18), medial age at diagnosis 2.0 years (0.1-17), 59% female and 67% family history of XLH. PHEX gene testing in 49 children (65%) of which 47 had a pathogenic mutation. Males were more likely to have bone and joint pain, kyphosis, tooth abscess and cransiosynostosis (p<0.05).

33 (45%) were managed with phosphate and calcitriol and 41 (55%) with Burosumab, which was associated with a higher serum phosphate level. Complications of phosphate and calcitriol therapy included nephrocalcinosis (32%), hyperparathyroidism (18%) and renal failure (1%).

Conclusion:

Prevalence of XLH in Australia is similar to other reported studies. XLH is associated with a significant burden of disease and males appeared more severely affected. Diagnosis is often delayed. Burosumab normalised serum phosphate in clinical use.

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Osteogenic potential of physical activities and their associations with bone mass in young adults from the Raine Study

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Physical activity questionnaires utilised in research generally assess metabolic equivalents of task (METs). It is unclear whether mechanical loading during physical activity, estimated from METs-based questionnaires, is associated with skeletal health. This cross-sectional study investigated how physical activity of high loading intensities and rates, assessed at ages 17- and 20-years, (a) compares with physical activity measured in METs, and (b) is associated with bone mass at age 20 years. 826 participants from the Raine Study Gen2 were assessed for physical activity energy expenditure over the past week via the International Physical Activity Questionnaire (IPAQ) at age 17- and 20-years. Loading scores (the product of peak force and application rate of an activity) per week were subsequently estimated from the IPAQ. Whole-body and appendicular bone mineral density (BMD) at age 20-years were assessed by dual-energy X-ray absorptiometry. Bland-Altman minimal detectable difference for physical activity Z-scores at age 17- and 20-years were 1.59 standard deviations (SDs) and 1.33 SDs respectively; greater than the *a priori* minimal clinically important change of 0.5 SDs. Loading score at age 17- and 20-years, but not IPAQ score, had significant positive associations with whole-body and leg BMD after adjustment for covariates (β=0.008 and 0.012g/cm^2 respectively). IPAQ score at age 20-years, but not loading score, had a significant positive association with arm BMD (β=0.007g/cm^2). There was no significant association between 3-year change in IPAQ or loading score and bone mass. This study revealed disagreement in associations of self-reported METs and loading score estimates with bone health in young adults. Participation in physical activity with higher loading scores was associated with whole-body and leg BMD, while higher energy expenditure was associated with arm BMD. Coupling traditional energy expenditure questionnaire outcomes with bone-loading estimates may improve understanding of the location-specific skeletal benefits of physical activity in young adults.

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A systematic review and meta-analysis of the effects of impact exercise on bone microarchitecture across the lifespan

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Background: Moderate- to high-impact exercise improves bone density across the lifespan, but effects on bone microarchitecture are unclear. This systematic review and meta-analysis investigated the effects of impact exercise on bone microarchitecture ranging from childhood to older age.

Methods: Four databases (PubMed, Embase, SportDiscus, Web of Science) were searched for randomised controlled trials comparing the effect of impact exercise with ground reaction forces equal to, or greater than, running, with no exercise. Bone parameters were measured by computed tomography or magnetic resonance imaging at the tibia, radius, lumbar spine and femur. Percentage change in bone parameters was compared between groups using mean differences (MD) and 95% confidence intervals calculated via random effects meta-analyses. Subgroup analyses for children and young adults, adults, postmenopausal women and older men were performed.

Results: Twenty-eight studies (n=2,789) were included in the meta-analysis. Impact exercise significantly improved total volumetric bone mineral density (vBMD) (MD: 0.79%, 95%CI: 0.40-1.19%), trabecular vBMD (0.78%, 0.33-1.23%), trabecular area (1.39%, 0.28-2.49%) and trabecular bone volume fraction (0.72%, 0.18-1.26%) at the distal tibia, and cortical vBMD (0.22%, 0.01-0.43%) at the tibial shaft. Similar trends for improvements in total vBMD and trabecular vBMD and area were observed at the radius. In our subgroup analyses, impact exercise significantly improved total vBMD (0.51%, 0.08-0.93%) and trabecular vBMD (0.82%, 0.20-1.45%) at the distal tibia in postmenopausal women. In children and young adults, a significant exercise effect was observed for total vBMD at the distal tibia (MD: 0.73%, 0-1.46%), and cortical vBMD (1.59%, 0.84-2.35%) and cortical area (5.12%, 1.55-8.69%) at the radial shaft. Impact exercise had no effect on bone parameters at the lumbar spine or femur.

Conclusions: Impact exercise influences cortical and trabecular compartments differently across the lifespan. Methodological inconsistencies among relatively low number of trials utilising three-dimensional skeletal imaging techniques emphasises the need for additional trials.

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Audit of Osteoporosis Management and Fracture Rates in Adult Lung Transplant Recipients

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Lung transplantation (LTx) requires long term immunosuppression with high dose glucocorticoids. At our institution, we have implemented a protocol of regular zoledronic acid (ZA) infusions for all LTx recipients from the time of wait-listing, with the aim of minimising glucocorticoid-induced osteoporosis and fracture. We sought to determine the prevalence of and risk factors for fracture, as well as rates of antiresorptive use and treatment-related adverse events in our patient cohort. Adults who underwent LTx at our institution from 1/1/2012 to 31/12/2018 and survived more than 6 months were included. Patients who moved interstate were excluded, leaving a total of 405 patients (168 female), with median age at LTx of 59 years.

A total of 73 patients (18%) sustained osteoporotic fractures, of whom 38 had fractured prior to LTx, and 51 (13%) developed new osteoporotic fractures post-LTx. The commonest fracture site was vertebral. There was also a high prevalence of non-osteoporotic fractures (n= 78, 19%), involving the ribs (n=49), foot (n=31) and ankle (n=15).

165 patients (41%) received at least 1 dose of ZA prior to LTx, and 346 (85%) received at least 1 dose of ZA post-LTx. Adverse events were uncommon. Atypical femoral fracture occurred in 1 patient (who had received 6 doses of zoledronic acid) and another developed osteonecrosis of the jaw.

Statistically significant risk factors for osteoporotic fracture post-LTx were: osteoporotic fracture pre-LTx, female gender and increasing age. Of note, bisphosphonate use pre-Tx was associated with increased fracture rates, likely representing selection bias. Following LTx, bisphosphonate therapy was not associated with osteoporotic fracture. This may reflect its frequent use.

In summary, although LTx recipients have a high prevalence of osteoporotic fractures, rates of post -LTx fracture may be lower than expected for such a high risk group, possibly as a result of routine intervention.

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Identification of asymptomatic vertebral fracture: A novel shape-based algorithm

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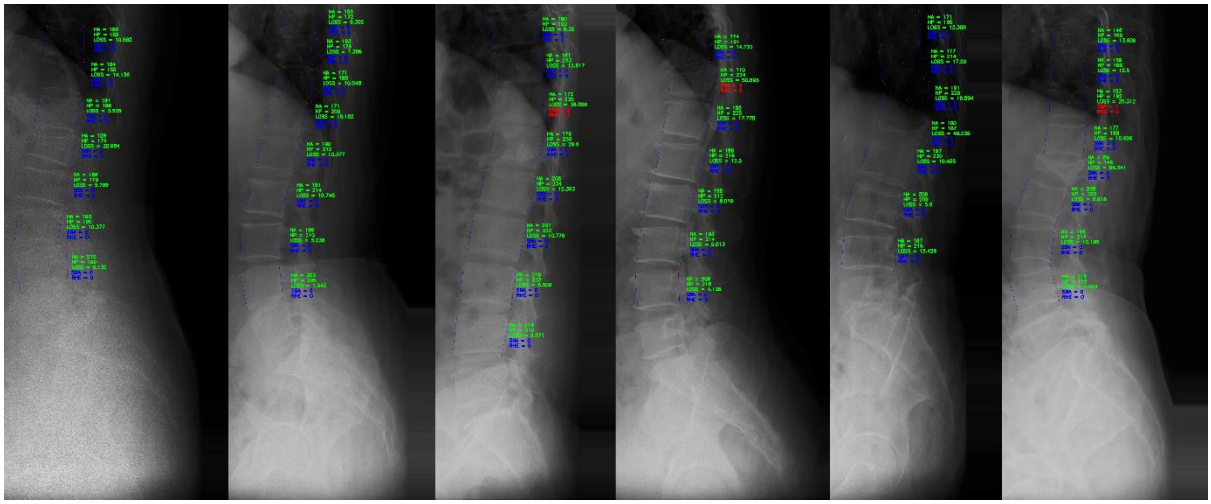
Background: Most vertebral fractures are asymptomatic, with more than two-thirds of vertebral fractures are undiagnosed. We sought to develop an automated shape-based algorithm to identify asymptomatic vertebral fractures.

Aims: To quantify the accuracy of the algorithm in the identification of vertebral fractures in men and women.

Methods: The study involved 106 individuals (109 lateral spinal X-rays), among whom 53 patients were clinically diagnosed by a rheumatologist to have a fracture. A shape-based algorithm was designed to identify 4 vertices in the segmented vertebra according to its contour and centroid by maximising the area formed by the vertices; anterior and posterior heights were then calculated for classification based on Genant's classification system. The accuracy of the algorithm was assessed in terms of sensitivity and specificity using the clinician's diagnosis as standard.

Results: The mean (SD) age of the 106 individuals was 57.1 (10.6 years). Among whom, 48 were diagnosed to have a fracture, and the algorithm identified 46, a sensitivity of 96%. Among the 61 without a fracture, the algorithm identified 19 as having a fracture, making the false positive rate of 31%. Among the 663 vertebrae examined, 55 were diagnosed to have a fracture, and the algorithm identified 45 (sensitivity of 82%). Among the 608 vertebrae without a fracture, the algorithm identified 67 as having a fracture (false positive of 11%). Further analysis of concordance between the Genant's classification and the algorithm scores showed that the accuracy was good for non-fracture (concordance of 98%) and severe fracture (43%), but not for mild and moderate fracture (17%).

Conclusion: The automated shape-based algorithm has good sensitivity and specificity for identifying asymptomatic vertebral fractures. The algorithm can help clinicians to screen a large volume of lateral spinal X-rays in clinical settings.



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Pain phenotype and risk of incident fractures over 10.7 years

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Objective Pain experience is heterogenous and linked to increased risk of fractures. Pain heterogeneity reflects that pain population consists of different subgroups of which the risk of fractures may differ. We sought to compare whether incident fractures risk over 10.7 years are different among three knee pain subgroups/phenotypes we previously identified.

Methods A total of 1099 participants (mean age 63 years) from a population-based cohort study were studied at baseline and followed up at 2.6 (n=875), 5.1 (n=768) and 10.7 years (n=563). Using latent class analysis that considered pain-related factors (i.e., sex, body mass index, emotional problems, education level, comorbidities, number of painful sites and MRI-detected knee structural damage), three knee pain phenotypes were previously identified: Class 1: high prevalence of emotional problems and low prevalence of structural damage (26%); Class 2: high prevalence of structural damage and low prevalence of emotional problems (20%); Class 3: low prevalence of emotional problems and low prevalence of structural damage (54%). Pain severity in the Class 1 and Class 2 was greater than that in Class 3. Fractures were self-reported, and falls risk was measured using the Physiological Profile Assessment.

Results There were 6 new hip, 19 vertebral, and 126 non-vertebral fractures during 10.7-year follow-up. Compared with Class 3, Class 1 had a higher risk of vertebral (risk ratio (RR)=3.32, 95%confident interval (CI): 1.04-10.63) and non-vertebral fractures (RR=1.47, 95%CI:1.04-2.07) after controlling for covariates, bone mineral density and falls risk. Participants in Class 2 also had a higher risk of vertebral fracture relative to those in Class 3 (RR=3.90, 95%CI: 1.02-14.84), but not non-vertebral fracture. No risk difference in hip fractures was found between classes.

Conclusion Class 1 and Class 2 had a higher risk of incident fractures than Class 3, highlighting that targeted preventive strategies for fractures are needed in pain population.

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Cognitive ability should be considered when assessing skeletal muscle strength and performance

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Aim: Assessment of muscle strength and performance require input from the person being measured. None of the recent operational definitions for sarcopenia that involve these assessments has considered the person's cognitive ability. We aimed to compare the muscle function components of the revised European Working Group on Sarcopenia in Older People (EWGSOP2), the Foundation for the National Institutes of Health (FNIH) and the Sarcopenia Definition and Outcomes Consortium (SDOC) algorithms for individuals with and without low-cognition.

Methods: This cross-sectional study involved 327 men (60-96yr). Global cognition was assessed using the Mini Mental State Examination (MMSE); MMSE scores <27 were considered as low-cognition. Handgrip strength (HGS) was measured by dynamometry and muscle performance by Timed Up-&-Go (TUG). Height and weight were measured and body mass index (BMI) calculated (kg/m^2). Chi-squared test (employing Fisher's exact test if expected cell count <5) identified differences in proportions and logistic regression models identified poor muscle function in association with low-cognition.

Results: Fifty-four (16.5%) men had low-cognition. The proportions of men with low-HGS were greater for those with vs without low-cognition according to different criteria: EWGSOP2 (9.3% vs 0.7%, p=0.002), FNIH (7.4% vs 0.7%, p=0.008) and SDOC (52.4% vs 38.1%, p=0.008); and low-HGS/BMI (18.5% vs 5.5%, p=0.003). Slow-TUG followed the same pattern (11.1% vs 1.1%, p<0.001). In models adjusted for age, men with low-cognition were 3-7 fold more likely to have low-HGS by EWGSOP2 (OR 6.66,

95%CI 1.18-37.8, p=0.03), FNIH (OR 5.71, 95%CI 0.93-35.0, p=0.06) and low-HGS/BMI (OR 3.01, 95%CI 1.19-7.63, p=0.02); and 6-fold more likely to have a slow-TUG (OR 5.82, 95%CI 1.31-25.8, p=0.02). The association between low-cognition and low-HGS by SDOC criteria was explained by age (OR 1.30, 95%CI 0.68-2.49, p=0.4).

Conclusion: Operational definitions for sarcopenia should consider low cognitive ability, at least in men, at the time muscle strength and performance are evaluated.

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Fracture incidence post lung transplantation

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Osteoporosis is common post lung transplantation but data are limited on subsequent fracture risk.

Aim: To determine incidence of fracture in lung transplant recipients.

Methods: Retrospective cohort study of lung transplant recipients listed between April 2014 and September 2015 at St Vincent's Hospital, Sydney and followed up to December 2020.

Results: The cohort consisted of 64 patients (35 females, 17 post-menopausal), 88% Caucasians with a mean age 48 ± 14.3 at time of transplant. During 245 person-years of follow-up, 17 patients (26.%) experienced at least one minimal trauma fracture yielding a rate of 69 fractures/1000 person-years (95% CI, 43 – 112). Crude rates were similar for women and men, however, after age-adjustment there was a non-significant trend towards a higher rate in women [HR 1.24 (0.46 - 3.36)] (figure).

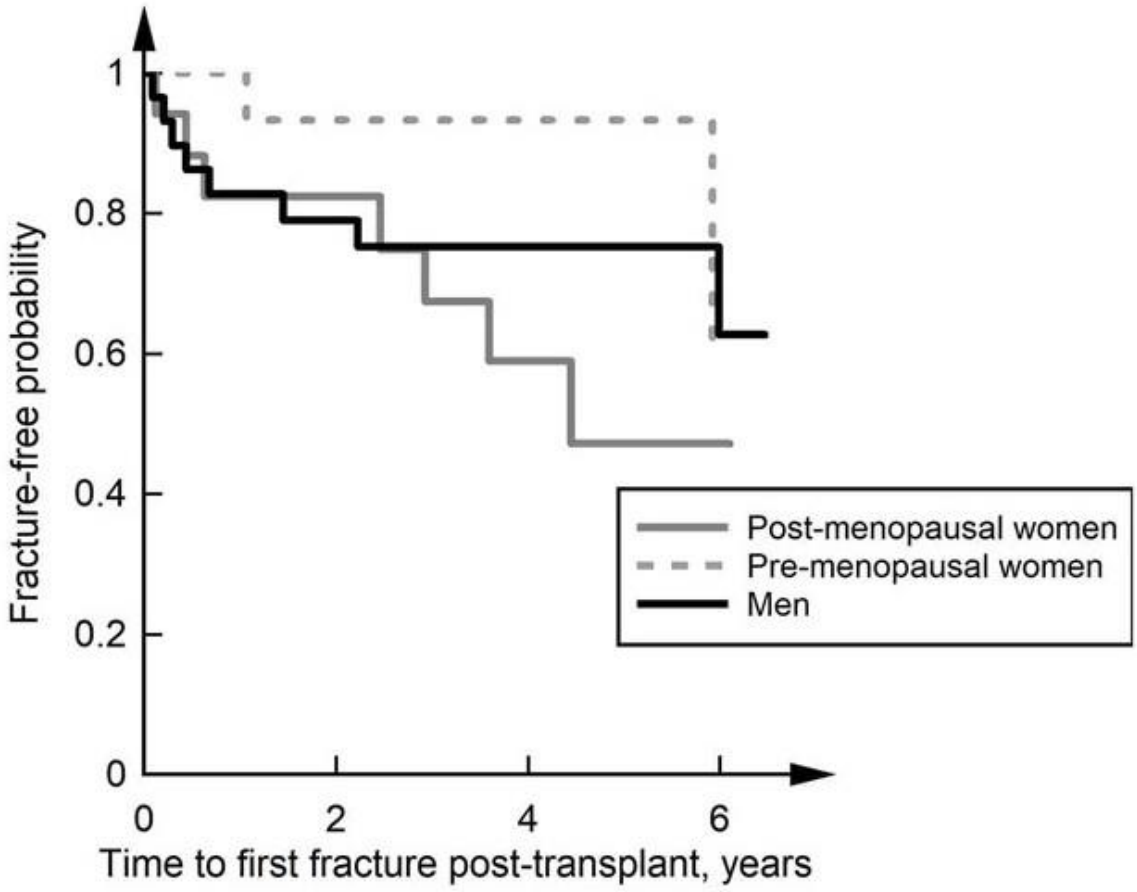
A total of 53 fractures was recorded, with 11 patients experiencing multiple fractures either occurring concurrently and/or subsequently. The majority of fractures were vertebral (62%), followed by rib (20%) and most were symptomatic (66%). Of the 18 asymptomatic fractures, 16 were vertebral and 2 were rib fractures. The mean time to first fracture was 2.1 ± 1.4 years, with almost half (47%) occurring in the first year post transplant.

Women who fractured (n=9) were significantly older, had lower BMD, more likely to have COPD, a history of smoking and to be post-menopausal. For men (n=8), presence of cystic fibrosis and lower BMI were associated with a non-significant trend towards increased fracture risk.

The majority (73%) received anti-resorptive treatment before and/or after transplantation, including 11 of the 17 (65%) patients who subsequently had fractures.

Conclusions: One in 4 of these lung transplant recipients suffered low trauma fractures; most commonly vertebral crush fractures. To our knowledge, this is the first study to quantify fracture risk up to 6 years after lung transplantation.

Figure: Kaplan-Meier fracture-free probability for lung transplant recipients according to gender and menopausal status



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Clinical and radiologic responses of central giant cell granuloma to denosumab: a 6-year observational cohort study

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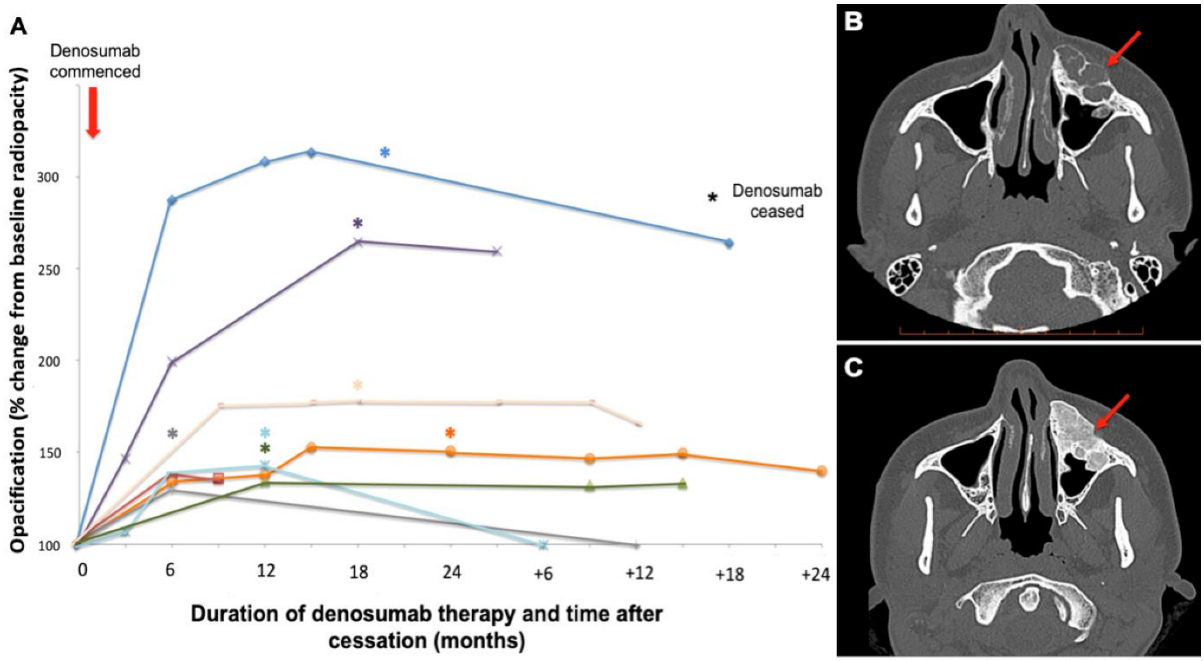

Figure 1: Radiological response of central giant cell granuloma to denosumab. Progressive opacification with duration of denosumab therapy for all patients (A) and computed tomography demonstrating lucent left maxillary lesion pre-denosumab (B) and opacification after four doses of denosumab (C).

Background:

Central giant cell granuloma (CGCG) is a rare tumour of the jaw occurring in young adults. Surgery is associated with high morbidity and recurrence rates. Denosumab is effective targeted therapy in a related but distinct, more aggressive entity, giant cell tumour of bone (GCTB). Experience in CGCG, a more indolent condition, is limited.

Aims:

To evaluate the safety and efficacy of denosumab in the management of CGCG, and recurrence risk post-cessation, using a more conservative treatment and monitoring regimen than used for GCTB.

Methods:

In this observational cohort study of all denosumab-treated CGCG at a tertiary referral centre (2015-2021), patients received denosumab 120mg using a modified regimen with less frequent dosing than used for GCTB to reduce the risk of adverse effects. Patients were followed for up to 72 months with standardised, low-radiation protocol: 3-monthly clinical, biochemical and radiological assessment (orthopantomogram +/- cone beam CT). Responses, complications and recurrence rates were evaluated.

Results:

Eight patients, median age 21 [IQR 6] years, received denosumab, median initial course 13 [10] doses. Responses were seen after 5.5 [4.5] doses (Fig.1): radiopacification representing intralesional ossification in all and radiological size reduction in three. Response was variable. Recurrence post-discontinuation occurred in 4 of 7 completing therapy after 12 [6.5] months. Larger baseline size, aggressive subtype and initial treatment <12 doses predicted recurrence. There was no osteonecrosis of the jaw and hypocalcaemia occurred in one receiving modified treatment regimen. Post-denosumab cessation rebound hypercalcaemia was mild and self-limiting in adults.

Conclusions:

This is the largest cohort of a diverse population of denosumab-treated CGCG and demonstrates the efficacy of denosumab at more conservative dosing than used in GCTB. Recurrence was predicted by larger baseline size, aggressive subtype and short initial treatment. This study supports the use of modified-dose denosumab in CGCG. Long-term low-dose radiological monitoring is required.

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Abdominal aortic calcification, bone mineral density and fractures: a systematic review and meta-analysis of observational studies

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Introduction: Abdominal aortic calcification (AAC) has been associated with poorer bone health and increased fracture risk in varied clinical settings, often in small studies with inconsistent findings. We aimed to synthesise observational studies on the association of AAC with bone mineral density (BMD) and fractures in a systematic review and meta-analysis.

Methods: Articles that reported on associations of AAC with BMD and/or fracture were retrieved from online databases from inception to August 2020. AAC was categorised as any/advanced AAC versus low/no AAC (reference group). The relationships between AAC and BMD at skeletal sites were determined by standardised mean difference [SMD] with 95% confidence intervals [CI]. To determine the association between AAC and risk and risk of any fracture, relative risk [RR] with 95%CI was calculated. Random effects models were applied.

Results: 79 articles (40 providing data for meta-analysis) were included from 2173 articles screened. Moderate quality evidence suggests BMD is lower at the total hip [SMD: -1.05, 95%CI (-1.47 to -0.63); I2 =94%; n=20277], femoral neck [SMD: -0.25 (-0.46 to -0.04); 99%; n=6981] and lumbar spine [SMD: -0.67 (-1.21 to -0.12); 99%; n=17260] in individuals with any/advanced AAC. Moderate quality evidence suggests fractures were more prevalent in individuals with any/advanced AAC [n=3515/14894 versus 2646/14730 (low/no AAC); RR: 1.66 (1.43 to 1.93); 90%]. In prospective studies, any/advanced AAC increased the risk of incident fractures [n=1197/6797 versus 1635/10113; RR 1.40 (1.22 to 1.61); 63%] with lower heterogeneity than seen for prevalent fracture. Findings were similar according to study location and imaging modality, but effects were more pronounced in older women.

Conclusion Moderate quality evidence supports the association of AAC with lower BMD and increased fracture risk. This suggests severe AAC is a risk factor for skeletal fragility and could be combined with BMD to enhance fracture risk prediction.

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Lower trabecular bone score bone mineral density and lean mass observed in women with premature ovarian insufficiency is prevented by oestrogen replacement

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Background

Low bone density (BMD) and fractures commonly affects women with premature ovarian insufficiency (POI). However, bone microarchitecture and body composition data are lacking.

Objective

Assess skeletal and soft tissue variables, fracture prevalence and oestrogen replacement therapy (ERT) effects in POI women.

Method

Cross-sectional and longitudinal studies of 60 normal karyotype POI women, aged 20-40 years, from 2005-2018. Dual x-ray absorpt

Table 1.
Baseline demographics, skeletal & body composition analysis with POI and controls.

		Control n=60	s-POI n=25	i-POI n=35	*P* Value
Age (years)		34 (30-38)	35 (29-37.5)	33 (30-38)	0.17
Ethnicity	Caucasian	98.3% (57)	68% (46)	82.9% (29)	**<0.001**
	Asian	0	20% (5)	17.14% (6)	NA
	Black	0	0	2.8% (1)	NA
	Hispanic	0	4% (1)	0	NA
BMI (kg/m$^{2)}$		25.84 (22.4-31.02)	24.80 (21.5-28.3)	26.50 (22.5-32.3)	0.252
Height (m)		1.66 (1.61-1.67)	1.58 (1.55-1.68)	1.61 (1.58-1.68)	.002
Fracture history		5 (8%)	5 (20%)	6 (17.6 %)	0.196
ERT use		NA	15 (68.2%)	29 (90.6%)	0.071
Bone Density					
BMD L1-L4(g/cm^2)		1.26 +/- 0.145	1.09 +/- 0.16	1.16+/- 0.14	**<0.001**
BMD NOF (g/cm^2)		1.04+/- 0.13	0.91 +/- 0.16	1.00 +/- 0.16	**0.002**
TBS					
TBS score		1.40 +/- 0.11	1.36 +/- 0.13	1.37+/- 0.10	0.213
Low TBS		12 (20%)	11 (44%)	6 (17.14%)	**0.031**
Body Composition					
Total lean mass (g)		42694 +/- 4902	36131+/ 5734	38343+/- 5134	**<0.001**
Lean mass index (g/m^2)		15465 (14183-16569)	13837 (12188-15092)	14107 (12978-15665)	**<0.001**
Total fat mass (g)		26856.5 (17904-40922)	20820 (18271-31845)	26178 (20610-46177)	0.138
Fat mass index (g/m^2)		15465 (14183-16569)	13838 (12188-15092)	14107 (12978-15665)	0.281

Data was expressed as mean (+/- SD), median (IQR) or n (%)
Boldface indicates *P* values that are statistically significant

iometry (DXA)-derived spinal (LS) and femoral neck (FN) BMD, trabecular bone score (TBS), total lean mass (TLM), total fat mass (TFM), and fracture prevalence were compared with 60 age-, sex- and BMI-matched population-based controls. Longitudinal changes in bone and body composition variables, and ERT effects were analysed using linear mixed models.

Results

POI participants were subdivided into spontaneous (s)-POI (n=25) and iatrogenic (i)-POI (n=35). Median (range) age of POI diagnosis was 34 (10-40) years with baseline DXA performed at median 1(0-13) year post-diagnosis. ERT was used by 81.5 % (similar for both groups). LS- and FN-BMD were lowest in s-POI ($p<0.05$). Low TBS (including both partially degraded and degraded TBS) [1] was more common in s-POI [(44%), $p=0.031$], versus other groups. TLM was lower in both s-POI and i-POI groups than controls ($p=0.001$). Fracture prevalence was not significantly different: 20%(s-POI), 17% (i-POI), and 8% (controls) ($p=0.196$). Longitudinal analysis of 24 POI women showed continuous ERT was associated with TLM increment of 256.5g/year ($p<0.001$) and protected against BMD loss. However, ERT interruption was associated with annual reductions in FN BMD and TBS of 0.020g/cm^2 and 0.007 ($p<0.05$), respectively.

Conclusion

Deficits in BMD, trabecular microarchitecture, and lean mass were present in women with POI. However, continuous ERT protected against declines in these variables, with an increase in TLM. Assessment of skeletal and muscle health, and advocating continuous ERT, is essential in POI women to optimise musculoskeletal outcomes.

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Recruitment of participants and early adherence to a digital health intervention for middle-aged and older women with osteoporosis

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Background: Remotely-delivered digital health interventions could support health behaviour change in middle-aged and older women with osteoporosis, and social media advertisements may be effective for recruiting participants to these interventions.

Methods: We are conducting a 6-month randomised controlled trial in 50 women aged ≥50 years currently prescribed anti-osteoporosis medication. Information (video/audio/text) on exercise, nutrition and medications for osteoporosis is delivered to participants via voice-activated Amazon Alexa Echo Show devices (intervention), or monthly emails (control). Facebook advertisements were targeted at users registered as women, aged ≥50 years and living in Australia, and these advertisements linked to a self-administered online form to initially assess eligibility. Potentially eligible respondents are being contacted for further screening and, if eligible, randomised to intervention or control following baseline assessments. Intervention group participants are provided with an Alexa device and instructed on using their voice to activate Alexa sessions on three days per week, as well as using natural language to respond to Alexa-delivered information and questions.

Results: Facebook advertisements costing $AUD1,000 were viewed by 60,425 users over one month (mean 2.1 times per user) and attracted 2,693 link clicks ($0.37 per click). Eligibility forms were completed by 207 women across all states and territories except Northern Territory, and 141 (68%) were potentially eligible and are being further screened. As of 15/8/2021, 40 women have enrolled and 22 (mean±SD age 65.7±7.4 years) have completed baseline assessments. Twelve women randomised to intervention to date have activated a total of 98 Alexa sessions (100% adherence) and provided a total of 1015 voice responses while interacting with Alexa-delivered exercise, nutrition and medication information.

Conclusions: Social media advertisements were cost-effective for recruiting middle-aged and older Australian women with osteoporosis into a remotely-delivered digital health trial. Initial data suggest Alexa is acceptable for delivering and collecting health behaviour information in this population.

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The relationships between muscle mass and function with bone remodelling markers in older adults: effects of acute aerobic and resistance exercises

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Purpose: Age-related muscle mass/strength loss affects independence and quality of life. Bone-muscle crosstalk is potentially mediated by bone remodelling markers (BRMs) including osteocalcin (OC). We tested the hypothesis that BRMs are correlated with baseline muscle mass/function which would predict BRM-responses after acute exercise. We also assessed the relationship between BRMs and insulin resistance (HOMA-IR).

Methods: Thirty-five older adults (25 women/10 men, 72±6 yrs) participated. Baseline assessments included body composition (DXA), muscle strength (grip, leg press) and physical performance (PPT, timed-up-and-go; gait speed, stair ascend/descend). Leg muscle quality (LMQ=leg press/leg lean mass) and stair climb power (SCP=force x velocity) were calculated. Participants performed (randomised) 30 mins aerobic (cycling 70%HR_{Peak}) and resistance exercise (leg press 70%RM, jumping). C-terminal telopeptide of type I collagen (CTX), procollagen of type I propeptide (P1NP), total (t)OC, undercarboxylated (uc)OC, glucose, insulin and HOMA-IR were assessed pre/post-exercise. Data was analysed using linear mixed models and beta-regressions.

Results: No difference in BRMs-responses to AE and RE, therefore data analysed together. Poorer PPT was related to lower baseline ß-CTX, P1NP and ucOC (all p<.05). Higher strength (LMQ, grip and leg) was related to higher baseline P1NP (all p<.05). Exercise decreased ß-CTX, tOC, insulin and HOMA-IR (all p<.05). ucOC remained unchanged. Participants with higher baseline muscle strength (SCP, LMQ, leg and grip) had lower post-exercise ß-CTX and tOC (all p <.05). Higher baseline ß-CTX, P1NP, tOC and ucOC was associated with lower post-exercise insulin resistance (HOMA-IR) (all p<.05).

Conclusions: Older adults with higher baseline BRMs are linked to greater muscle function and lower insulin resistance. Acute exercise decreases ß-CTX and tOC, and higher baseline muscle strength was related to lower responses of these specific BRMs. Despite mechanisms behind the specific component of bone-muscle crosstalk remaining unclear, BRMs may be used to identify individuals with poorer muscle function and insulin sensitivity.

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Effects of Acute Exercise on Bone Turnover Markers in Middle-Aged and Older Adults: a Systematic Review

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Purpose: Long-term exercise improves bone health eliciting anabolic effects if characterised by progressive, dynamic, novel patterns and applied rapidly. Acute-exercise effects with varying mechanical stimuli is less clear. Bone turnover markers (BTMs), surrogate measures of bone health, are used to measure acute exercise-responses, but findings are contradictory possibly owing to factors (feeding, circadian-effects) modulating responses. This systematic review examines the effects of acute aerobic (AE), resistance (RE) and impact exercises on BTMs in middle and older-aged adults.

Methods: We searched PubMed, SCOPUS, Web of Science and EMBASE up to 22nd April 2020. Eligibility criteria: randomised controlled trials (RCTs) and single-arm studies; middle-aged (50 to 65 years) and older adults (>65 years); a single-bout, acute-exercise (AE, RE, impact) intervention with measurement of BTMs. PROSPERO registration number CRD42020145359

Results: Thirteen studies were included; 8 in middle-aged (n= 275, 212 women/63 men, mean age= 57.9 ± 1.5 years) and 5 in older-adults (n= 93, 50 women/43 men, mean age= 68.2 ± 2.2 years). Eleven studies included AE (7 middle-aged/4 older-adults), and two included RE (both middle-aged). AE significantly increased C-terminal telopeptide (CTX), alkaline phosphatase (ALP) and bone-ALP in middle-aged and older-adults. AE also significantly increased total osteocalcin (tOC) in middle-aged men and Procollagen I Carboxyterminal Propeptide and Cross-Linked Carboxyterminal Telopeptide of Type I Collagen in older women. RE alone decreased ALP in older-adults. In middle-aged adults, RE with impact had no effect on tOC or BALP, but significantly decreased CTX. Impact (jumping) exercise alone increased Procollagen Type 1 N Propeptide and tOC in middle-aged women. Quality assessment results identifies a lack of RCTs, low quality evidence, small sample sizes and large variance in protocols.

Conclusions: Acute exercise is an effective tool to modify BTMs, however, responses appear to be exercise modality-, intensity-, age- and sex-specific. Higher quality and larger RCTs are needed.

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Changes in body composition in the year following critical illness

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OBJECTIVES: Muscle loss during critical illness is well documented. The recovery of muscle loss in survivorship and its role in physical disability is not described. Our aims were to measure changes in dual x-ray absorptiometry (DXA) estimated lean and fat mass in Intensive Care Unit (ICU) survivors in the year following critical illness and compare to population controls. Secondary aims included examining the association between body composition and health-related quality of life (HrQOL).

METHODS: Using prospective observational data, we estimated spine and hip DXA derived lean and fat mass at discharge and 1-year follow up in adult ICU patients who underwent mechanical ventilation for at least 24 hours. Annualized total and percentage changes in lean and fat mass were compared to age-sex-height matched controls from the Geelong Osteoporosis Study (GOS) via multivariable linear regression analysis. HrQOL was measured via the EuroQOL-5D-3L and Pearson's correlation coefficient used to determined its relationship with body composition.

RESULTS: Sixty-four cases were included, with median age 68.8yr [IQR60.8, 74.6], ICU length of stay 6.5d [IQR 4, 9] and duration of mechanical ventilation 87hrs [IQR 47, 143]. ICU survivors demonstrated greater annual increases in lean (+2.30%, 95%CI 1.64-2.95; p<0.001) and fat mass (+13.07%, 95%CI 9.37-16.78; p<0.001) than controls. At 1-yr follow-up lean mass

values remained lower in the ICU group (-0.96kg, 95%CI -1.91 to -0.01; p=0.047). EuroQol measures at one-year were negatively correlated with fat (r=-.3) but not lean mass.

CONCLUSIONS: Mechanically ventilated adult ICU patients gained lean mass in the year following critical illness but did not reach the level of matched population-based controls. Fat mass was greater in ICU pts than controls and displayed negative correlation with HrQOL. Understanding the factors associated with, and effect of increasing muscle mass and reducing fat mass in the year after critical illness requires further investigation.

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Effect of nutritional calcium and phosphate loading on calciprotein particles

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Background: Calciprotein particles (CPP) are endogenous, colloidal aggregates of calcium and phosphate and the mineral-binding protein fetuin-A. Serum CPP have been reported to be elevated in multiple conditions, including chronic kidney disease (CKD), where levels are associated with increased cardiovascular risk. Dietary phosphate loading in rodents leads to increased circulating CPP levels, but the effect of nutritional loading in humans has not been previously described.

Aim: To examine the effect of nutritional loading on serum CPP in participants with and without CKD.

Methods: Amorphous calcium phosphate containing primary CPP (CPP-I) and hydroxyapatite-containing secondary (CPP-II) were measured by flow cytometry in 14 patients with CKD (eGFR<60mL/min/1.73m^2) and 16 age- and gender-matched healthy adults. Serum CPP were measured after an overnight fast and then at five timepoints after participants received a standardised meal (Sanitarium Up&Go; 250mL, containing 300mg calcium [38% RDI] and 188mg phosphate [19% RDI]). Linear mixed effects models were fitted to examine differences between groups at each timepoint, and to estimate the postprandial excursion of serum CPP from baseline values.

Results: Mean age of the cohort was 44 years and 57% were female. The median eGFR of the CKD cohort was 28.5mL/min/1.73m^2 (range 10-51mL/min/1.73m^2). There were no between-group differences in serum CPP-I or CPP-II at any timepoint. The post-prandial excursion of serum CPP was subsequently examined after combining the groups (Table 1). Serum levels of CPP-I and CPP-II rose significantly after the standardised meal. Levels of both CPP-1 and CPP-II remained elevated from baseline values at four hours after the meal.

Conclusions: We found a significant acute post-prandial effect on serum CPP-I and CPP-II confirming dietary mineral as an important modifier of circulating CPP levels. In this small study, CKD did not significantly affect the post-prandial excursion of serum CPP.

Table 1: Serum calciprotein particles (CPP) after fasting and standardised meal

	Median (IQR)[a]	Change from baseline (95% CI)[b]	p-value
CPP-I (x100 particles/mL)			
Fasting baseline	9.3 (4.0, 16.3)		
30 minutes post meal	8.7 (2.7, 26.0)	70.3% (-0.2 to 190.7)	0.05
60 minutes post meal	34.7 (15.3, 136.7)	686.2% (353.9 to 1261.9)	<0.01
120 minutes post meal	60.7 (38.7, 172.0)	1199.4% (626.5 to 2224.1)	<0.01
180 minutes post meal	78.0 (34.7, 151.3)	1174.9% (583.3 to 2278.5)	<0.01
240 minutes post meal	42.0 (17.3, 91.3)	543.5% (228.0 to 1162.6)	<0.01
CPP-II (x100 particles/mL)			
Fasting baseline	1.3 (1.3, 3.3)		
30 minutes post meal	2.0 (1.3, 6.0)	56.2% (3.3 to 136.2)	0.03
60 minutes post meal	8.0 (5.3, 14.0)	345.8% (194.7 to 574.5)	<0.01
120 minutes post meal	20.7 (10.7, 32.7)	872.0% (537.4 to 1382.1)	<0.01
180 minutes post meal	17.7 (12.0, 32.7)	777.0% (469.0 to 1251.7)	<0.01
240 minutes post meal	13.0 (8.7, 18.7)	529.3% (302.9 to 883.0)	<0.01

[a]Serum CPP levels are expressed as median (25^{th}, 75^{th} percentiles).

[b]Change from baseline of CPP-I and CPP-II was estimated by fitting linear mixed effects models. For these analyses CPP-I and CPP-II were natural log transformed to ensure normal distribution of residuals. To aide in interpretation, coefficient estimates have been exponentiated and expressed as percentage and 95% confidence interval.

External validation of a risk assessment tool for predicting fragility fractures and mortality in the Osteoporotic Fractures in Men (MrOS) Study

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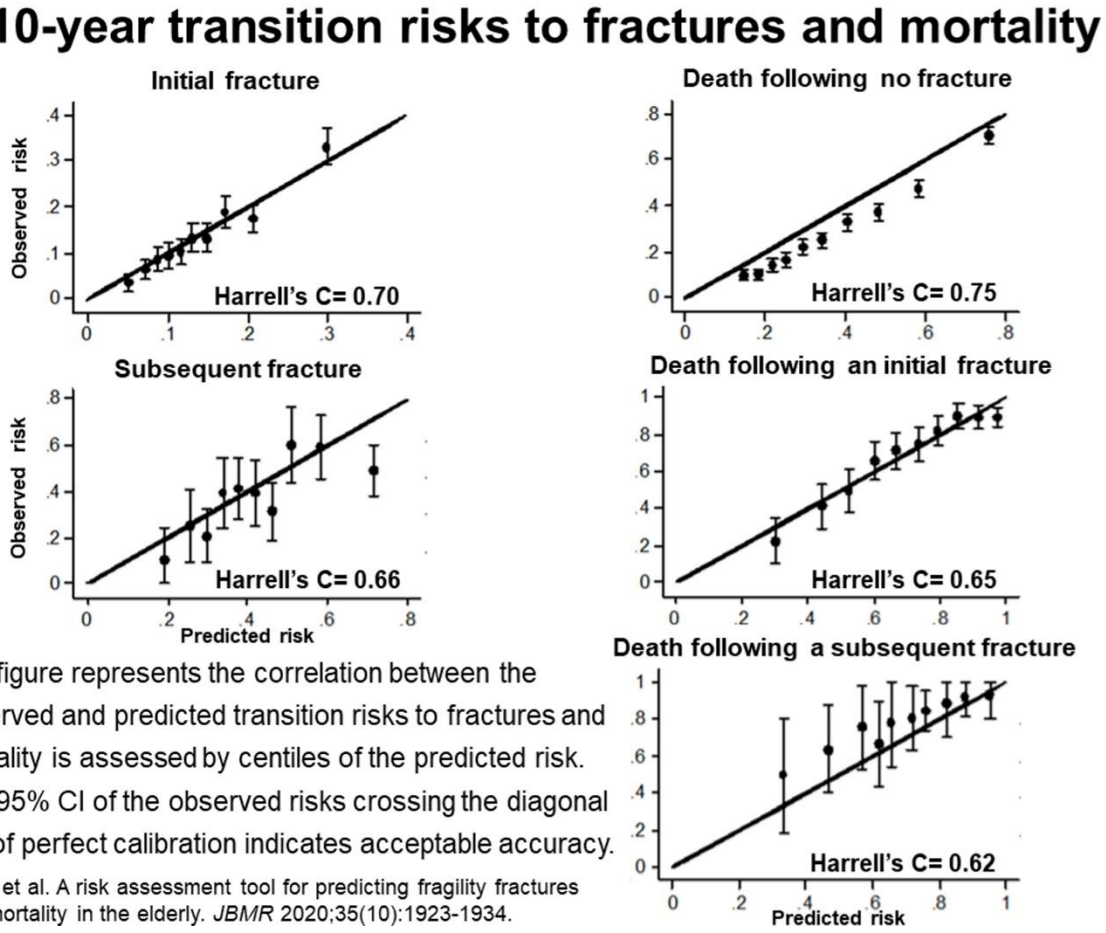
Background: Existing fracture prediction tools are not designed to predict fracture-related consequences. We have developed a risk assessment tool[1] to predict fractures and mortality using the Dubbo Osteoporosis Epidemiology Study and the Canadian Multicentre Osteoporosis Study. The tool requires external validation for widespread use.

Objective: to quantify the accuracy of the tool in predicting initial fractures, subsequent fractures and mortality in the MrOS USA cohort.

Methods: 5,994 MrOS men aged 74 (±5.9) years were followed for fractures and mortality. Baseline predictors included age, bone mineral density, prior falls, prior fracture, cardiovascular and respiratory diseases, diabetes, hypertension and cancer. Predicted 5- and 10-year transition risks to initial fractures, subsequent fractures and mortality were estimated. Discrimination ability was assessed using the Harrell's C index, and calibration assessed by dividing the cohort into centiles of predicted risk and comparing the observed and predicted risk.

Results: During a median follow-up time of 14 years (IQR:8-17), there were 1,085 initial incident fractures (~15.5 fractures/1,000 person-years; 95%CI: 15.6-16.5) followed by 236 subsequent fractures (46.7; 40.9-53.1). Mortality rates among subjects with no fracture, an initial and subsequent fracture were 4.8 deaths/100 person-years (95%CI: 4.6-4.9), 11.8 (10.4-12.7), and 19.4 (16.5-22.6), respectively. Baseline predictors remained significant in the MrOS cohort. The tool had moderate discrimination ability with the highest concordance documented for predicting deaths (C index: 0.75), any initial fracture (0.70) or hip fracture (0.74). Importantly, the tool accurately predicted transition risks to initial fractures, subsequent fractures and post-fracture deaths. However, it overestimated the risk to death from those not suffering fracture, possibly due to substantial between-cohort differences in baseline mortality risk (Fig).

Conclusions: The risk assessment tool provided moderate discrimination and accurate calibration for predicting fractures and fracture-related mortality in men. Subsequent validation in women is necessary prior to consideration of its use in clinical practice settings.



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Osteoporosis in patients with intestinal failure at Westmead Hospital: a 20-year single-centre review

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Background and Aim

Home parenteral nutrition (HPN) provides nutritional support to intestinal failure patients. The aetiology of HPN-related metabolic bone disorders are multifactorial, related to underlying malnutrition and malabsorption, hypercalciuria and aluminium toxicity in earlier preparations[1-3]. Recent studies, however have indicated improvement in bone mineral density (BMD) after commencement of HPN, with high prevalence of low BMD at baseline[1,4]. This 20-year retrospective study reviews baseline DXA characteristics of HPN patients at a single-centre Intestinal Failure Service.

Material and Methods

All HPN patients at Westmead Hospital between 2000 and 2020 were retrospectively identified to obtain baseline DXA results and demographics (n=59). After excluding subjects who did not have a baseline DXA scan (GE Lunar) at Westmead Hospital, 22 patients were reviewed.

Results

Mean age was 48.6 years (18-84), with 12 males (54.5%) and 10 females (45.5%). 13/21 patients (61.9%) had Vitamin D deficiency (< 50nmol/L) at HPN commencement and 2 (9.1%) were on glucocorticoids. At baseline there was a significant burden of osteopenia (11/22, 50%) and osteoporosis (5/22, 22.7%) in this cohort. The broad categories for HPN indication were prolonged bowel rest (n=8) and inadequate absorption (n=14) related to various pathologies including short gut syndrome, gastroparesis and enterocutaneous fistula. These groups did not differ in their baseline bone density (1.05g/cm^2 vs. 1.08g/cm^2, p=0.84) or Vitamin D level (48.3nmol/L vs. 47.2nmol/L, p=0.93). Fracture rates could not be assessed. Of the six patients who died from underlying disease processes, baseline BMD T-score ≤-2.5 was not associated with death during the study period (p=0.68).

Conclusion

Metabolic bone disease is common in intestinal failure patients undergoing HPN, compounded by high rates of Vitamin D deficiency. This study highlights the importance of recognising this at-risk group with a need for longterm surveillance protocol with serial DXAs, consideration of Vitamin D replacement and/or early anti-resorptive therapy.

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Eating disorders are associated with increased risk of fall injury and hip fracture in Swedish women and men

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Eating disorders such as anorexia nervosa and bulimia nervosa have been associated with decreased bone mineral density and increased fracture risk, but the association with fall injuries without fracture has not previously been investigated. Furthermore, due to a relatively low number of men in previous studies, fracture risk in men with eating disorders has been insufficiently studied.

In the present Swedish retrospective study, 8 867 patients (90.6% women) with a diagnosed eating disorder and 88 670 age, sex and county matched controls who had never been diagnosed with an eating disorder were investigated. The mean (standard deviation) age of the patients and controls was 41.6 (13.7) years and the follow-up time 9.6 (5.2-14.4) years (median, interquartile range) for patients and 10.1 (5.5-14.2) years for controls.

The proportions of injurious falls without fracture (17.3% vs. 9.0%) and of hip fracture (1.6% vs. 0.7%) were substantially greater in patients with an eating disorder than in their corresponding population controls. In unadjusted Cox proportional hazards models, individuals with an eating disorder had a higher risk of injurious falls without fracture (hazard ratio (HR), 95% confidence interval

(CI): 2.07, 1.96-2.18), and hip fracture (HR 2.30, CI 1.92-2.75) than the risk observed in the controls. The HR for any investigated outcome associated with an eating disorder did not differ by sex or age group (interaction term p>0.10). The risk of injurious falls without fracture and hip fracture was increased in both women (HR 2.07, CI 1.95-2.19 and HR 2.41, CI 1.98-2.93, respectively) and men (HR 2.09, CI 1.76-2.49 and HR 1.84, CI 1.12-3.02, respectively).

In conclusion, the risk of injurious falls without fracture and hip fracture is increased in both women and men with eating disorders, indicating measures to prevent both falls and fractures are important in these patients, regardless of age and sex.

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Osteoglycin across the adult lifespan

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Background: Circulating Osteoglycin (OGN), a proteoglycan is released from both bone and muscle and has been associated with markers of metabolic health. However, it is not yet clear whether the levels of circulating OGN change throughout the adult lifespan or if circulating levels of OGN are associated with metabolic markers or aerobic fitness levels. **Methods:** 107 individuals (46 males and 61 females) aged 21-87 years were included in the study. Serum OGN levels, aerobic capacity (VO_{2peak}), glucose and homeostatic model assessment for insulin resistance (HOMA-IR) were assessed. T-tests were used to compare participant characteristics between sexes. Regression analyses were performed to assess the relationship of OGN with age, aerobic fitness and metabolic markers. **Results**: OGN displayed a "U shaped" relationship with age in both males and females. However, males had higher absolute levels of OGN than females across the lifespan (β=0.23, p=0.03). Overall, age and sex explained 16 % of the variance in OGN (adjusted R^2 = 0.16; p < 0.001). Higher OGN was associated with higher VO_{2peak} (β = 0.02, p = 0.001) however those aged below 50 showed a stronger positive relationship than those aged above 50. A higher OGN level was associated with a higher circulating glucose level (β = 0.17, p < 0.01). No association was observed between OGN and HOMA-IR. **Conclusions:** OGN across the lifespan was characterized by a U-shaped curve and this was similar between sexes. Those with a higher aerobic capacity or higher baseline glucose concentration had higher OGN levels. Our data suggests an association between OGN and aerobic fitness and glucose regulation. Whether it plays a direct mediating role in either aerobic fitness or glucose regulation humans is still unknown.

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Vertebral Fractures following Interruption of Denosumab Therapy

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Objective:

To assess the incidence of new vertebral fractures in patients with interruption of denosumab therapy between January 2019 and April 2021 at Eastern Health.

Background:

FREEDOM extension trial demonstrated lower rates of vertebral and non-vertebral fractures in patients treated with denosumab therapy for up to 10 years[1]. Multiple case reports and a recent systematic review suggested increased risk of multiple vertebral fractures in patients when denosumab therapy was discontinued[2,3].

Method:

This was designed as a retrospective audit. All patients with a diagnosis of fracture who were taking denosumab between January 2019 and April 2021 were identified within the hospital database. For the purpose of this study, only patients with vertebral fractures were included. Medical record of each patient was reviewed to determine if vertebral fractures occurred in the setting of denosumab interruption. This was defined as interruption of delay of > 1 month. Data collected were entered into Microsoft Excel and data analysed using SPSS IBM 25.0. Student t-test was used to compare the differences of sample mean in both groups.

Results:

48 patients were included in this study. Of those 48 patients, 17 patients had interruption of denosumab therapy. Both groups had similar clinical characteristics at baseline. Incidence of new vertebral fractures was similar in those with and without denosumab interruption. Results are summarised in table 1.

Table 1. Baseline characteristics, risk factors for bone lose, incidence of vertebral fractures and serum biochemical markers. N= 48			
	Denosumab Interruption N=17 (35.4%)	Without Denosumab Interruption N= 31 (64.6%)	P-value
Gender (N/%)			
Female	14 (82.4%)	26 (83.9%)	0.89
Male	3 (17.6%)	5 (16.1%)	
Age (mean ± SD)	80.7 ± 8.9 years	82.0 ± 7.8 years	0.61
Corrected Calcium (mmol/L)	2.39 ± 0.21	2.36 ± 0.11	0.53
Phosphate (mmol/L)	1.16 ± 0.32	1.07 ± 0.26	0.36
Vitamin D (nmol/L)	94.9 ± 29.6	92.5 ± 34.8	0.82
Duration of Denosumab treatment in years (mean ± SD)	3.0 ± 1.75	2.3 ± 1.83	0.2
Previous osteoporosis treatments (N/%)	4 (23.5%)	7 (22.6%)	0.94
+ Alendronate	1 (5.9%)	4 (12.9%)	0.45
+ Risedronate	2 (11.8%)	1 (3.2%)	0.24
+ Raloxifene	0 (0%)	1 (3.2%)	0.45
+ Zoledronic acid	1 (5.9%)	0 (0%)	0.17
+ Strontium	0 (0%)	1 (3.2%)	0.45
Duration of previous osteoporosis treatments in years (mean ± SD)	2.63 ± 1.8	5.0 ± 2.2	0.09
Types of fracture			
+ Vertebral fractures	16 (94.1%)	25 (80.6%)	0.21
+ Other fractures	1 (5.9%)	6 (19.4%)	
Risk factors – at least 1 risk factors (N/%)	11 (64.7%)	9 (29.0%)	0.017
+ Use of steroids	6 (35.3%)	4 (12.9%)	0.068
+ Malnutrition	0 (0%)	2 (6.5%)	0.29
+ Thyroid disease	2 (11.8%)	1 (3.2%)	0.24
+ Parathyroid disease	1 (5.9%)	1 (3.2%)	0.66
+ Diabetes	0 (0%)	2 (6.5%)	0.29
+ Kidney disease	1 (5.9%)	0 (0%)	0.17

Conclusion:

In this single-centre retrospective audit, we observed a large proportion (35.4%) of vertebral fractures occurring in patients on denosumab occurred in those who had interrupted denosumab therapy. Steroid use may be an important co-factor in denosumab discontinuation and subsequent fracture. In order to determine whether denosumab interruption contributes to the risk of fracture, we would need to know the rate of interruption in the denosumab treated population. However, larger studies are required to confirm the findings.

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Determining need for a co-designed educational program to increase musculoskeletal health awareness among Aboriginal and Torres Strait Islander adults: findings from focus groups

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Background: Falls and fracture rates are higher among Indigenous Australians compared with non-Indigenous Australians. Knowledge and awareness about osteoporosis and its related risk factors are important contributors to fall and fracture prevention. No data exist on musculoskeletal (MSK) health knowledge among Indigenous Australians. We conducted a needs-analysis to identify knowledge gaps to then co-design an educational program with Community, for Community.

Methods: Two focus groups were undertaken with Indigenous Australians aged 35-75years residing in Victoria (n=2x5 participants). Each focus group was split into three sections: bone health, muscle health and practicalities. Each section was given an introduction by the facilitator, followed by semi-structured questions. Focus group recordings were transcribed, and thematic analyses performed using NVivo.

Results: Osteoporosis and osteoarthritis were used interchangeably suggesting a lack of awareness between the two. Diet (healthy eating), nutrition (calcium), vitamin D and exercise were identified as factors important for healthy bones. Weak muscles were identified to increase falls risk. Participants were unsure of whether chronic disease (cardiovascular disease, diabetes, and chronic kidney disease) impacted MSK health; due to personal experiences, the effects of cancer on MSK health were discussed by participants. Mental health was identified as a key factor that influenced all aspects of health and was highly recommended to be included in the educational program. All participants identified "face-to-face" sessions as the optimal delivery method for an educational program. As storytelling is a crucial element of Aboriginal culture, focus groups highlighted the imperative of including a community member in each session to tell a personal story related to MSK health.

Conclusions: The focus groups identified knowledge gaps in MSK health and indicated the need for a co-designed educational program. This should be culturally appropriate, delivered in a face-to-face setting and fulfil Community needs to effectively improve awareness of MSK health in Indigenous Australians.

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Bone mineral density in retired elite rowers

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Background: Young elite rowers accrue greater areal bone mineral density (aBMD) associated with mechanical loading. However, it is unknown whether retired elite rowers maintain a higher aBMD with ageing. Therefore, we measured aBMD in retired older elite male rowers.

Study design: Retired elite athletes were recruited from a database of athletes aged 45-80 years and who competed at State, National or International level for 10 or more years. Dual energy x-ray absorptiometry (DXA) measured aBMD (whole body, total hip and lumbar spine) and body composition where corrections for body size were made by dividing by height squared (kg/m^2) giving fat mass index (FMI) and appendicular lean mass index (ALMI). VO2max (mL/kg/min) was assessed to determine cardiorespiratory fitness. Fasting blood samples were collected to assess lipids and kidney function. Data are expressed as mean±SD; aBMD (T-scores) are reported. Associations between ALMI, FMI and VO2max with aBMD with age adjustments was determined and p-values reported.

Results: The 82 men were aged 63.6±8.6 years, weighed 88.5±12.4kg, with a height of 183.6±7.7cm. They had normal ALMI ($8.7±0.8kg/m^2$) and FMI ($6.6±2.3kg/m^2$), while VO2max was in the superior range (38.8±9.8ml/kg/min). Biochemical tests revealed healthy ranges for lipids (cholesterol: 5.0±1.2, triglycerides: 1.2±0.8, HDL: 2.1±1.1, LDL: 3.1±1.0) and kidney function (creatinine: 82.0±14.8, eGFR: 83.8±9.6). BMD T-scores were either normal or high-normal at all sites: whole body ($1.37±0.11g/cm^2$; T-score:+1.7), total hip ($1.08±0.13g/cm^2$; T-score:-0.11), femoral neck ($1.00±0.13g/cm^2$; T-score:-0.53) and lumbar spine ($1.16±0.14 g/cm^2$; T-score:-0.41). ALMI was positively associated with aBMD at all sites except lumbar spine (all $p<0.05$), FMI was positively associated with aBMD at all sites ($p<0.05$). There were negative associations between total hip aBMD and VO2max ($p=0.01$).

Conclusion: Older retired elite male rowers had normal or high-normal aBMD. Performing high intensity endurance exercise for ≥10 years at a younger age, may contribute to improved bone health with ageing.

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Bone Microarchitecture in Transgender Adults: a Cross Sectional Study

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Gender-affirming hormone therapy aligns physical characteristics with an individual's gender identity, but given estradiol regulates bone remodelling, may compromise bone morphology. We hypothesised that trans men receiving testosterone therapy for masculinisation, due to reduced estradiol concentrations, have compromised bone microarchitecture. In trans women receiving feminising hormone therapy to increase estradiol and lower testosterone concentrations, we hypothesised that bone microarchitecture would be preserved.

We compared distal radial and tibial microarchitecture using high-resolution peripheral quantitative CT images in a cross-sectional study of 41 trans men with 71 cisgender female controls, and 40 trans women with 51 cisgender male controls. Differences between groups were expressed as T-scores (standardized deviations (SD) from the mean) and 98% confidence interval given adjustment for multiple comparators.

Trans men had higher distal tibial total volumetric bone mineral density (vBMD), 0.71 SD (0.30, 1.12), p<0.01 relative to cisgender female controls with preserved cortical morphology and trabecular bone volume fraction. Trabecular number and separation were similar, but thickness was 0.50 SD higher (-0.08, 0.92), p=0.02 than cisgender female controls. Conversely, trans women had -0.55 SD lower distal tibial total vBMD (-1.01, -0.08), p=0.02 relative to cisgender male controls due to higher cortical porosity, 0.63 SD (0.19, 1.07), p=0.01 and lower trabecular bone volume fraction -0.57 SD (-1.05, -0.08), p=0.02. Trabeculae were fewer -0.47 SD (-0.94, 0.01), p=0.05, thicker 0.49 SD (0.01, 0.96, p = 0.04) but separation was not increased. Distal radius findings were similar.

Contrary to the hypotheses, bone microarchitecture was not compromised in trans men, perhaps because aromatisation of administered testosterone prevented bone loss. Trans women may not be protected from microarchitectural deterioration by estradiol administration, perhaps because the dose was insufficient to offset reduced aromatizable testosterone.

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To stop or not to stop: Denosumab in the octogenarian with renal impairment

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Hypocalcaemia is a known side effect of denosumab therapy. The risk increases as renal function decline, with incidence of up to 50% in CKD-4 patients (1). Since osteoporosis affects older population disproportionately, low eGFR is common amongst treated patients since eGFR declines with age (2). In acute renal failure, denosumab may be withheld to mitigate the risk of hypocalcaemia. However, its cessation can lead to high bone turnover and increased fracture risk (3). This poses a conundrum as to whether to continue denosumab and monitor for hypocalcaemia, or to discontinue and monitor for rebound high bone turnover.

We describe an 81-year-old female who presented with acute renal failure (eGFR = 12) from sepsis due to renal calculi. She was on denosumab 6-monthly since 2017 for osteoporosis and L4 fracture. Her inpatient ionized calcium was normal at 1.17 mmol/L (total calcium 2.06 mmol/L, albumin 22g/L). Denosumab was ceased post discharge due to hypocalcemic concerns. Eight months after last dose of denosumab, her bone specific alkaline phosphatase (bsALP) was elevated at 35.2 ug/L reflective of high bone turnover.

Hypocalcaemia is common in the elderly population during severe acute illness (4). It is uncertain whether patients on denosumab may be more prone to hypocalcaemia in the setting of concurrent acute illness. In an Austin Health audit of 108 subjects on established denosumab (≥ 2 doses), 27 (25%) subjects had hypocalcemia in routine or inpatient blood tests, only 3 subjects had eGFR < 35. Whilst subjects on established denosumab were reported to be less prone to hypocalcaemia in the setting of suppressed bone turnover (5), more studies are required in this area whereby age related hypoalbuminemia, differential protein binding with renal impairment, acidaemia during sepsis all pose challenges to calcium interpretation and management of ongoing denosumab treatment.

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Burosumab in Adult X-Linked Hypophosphatemia

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X-linked Hypophosphatemia (XLH) is a rare X-linked dominant disorder with complete penetrance, caused by loss-of-function mutations in PHEX gene, which encodes an enzyme that degrades osteopontin and suppresses fibroblast growth factor 23 (FGF23)[1,2]. Treatment option for adults was limited to phosphate-calcitriol therapy that is poorly tolerated and does not address the underlying pathophysiology of XLH.

In 2018, a randomized controlled trial (RCT) assigned adults between 18 and 65 years of age with confirmed PHEX mutation to receive either burosumab, a monoclonal IgG1 antibody that targets FGF23 expression, at 1mg/kg every 4 weeks (burosumab-burosumab group) or placebo for 24 weeks3. This was followed by open-label burosumab to all subjects for 24 weeks (placebo-burosumab group). Primary analysis at week 24 shows 94.1% participants in the burosumab-burosumab group attained normal phosphate level and greater fracture healing compared to control[3,4]. Unfortunately, this medication is not widely available yet.

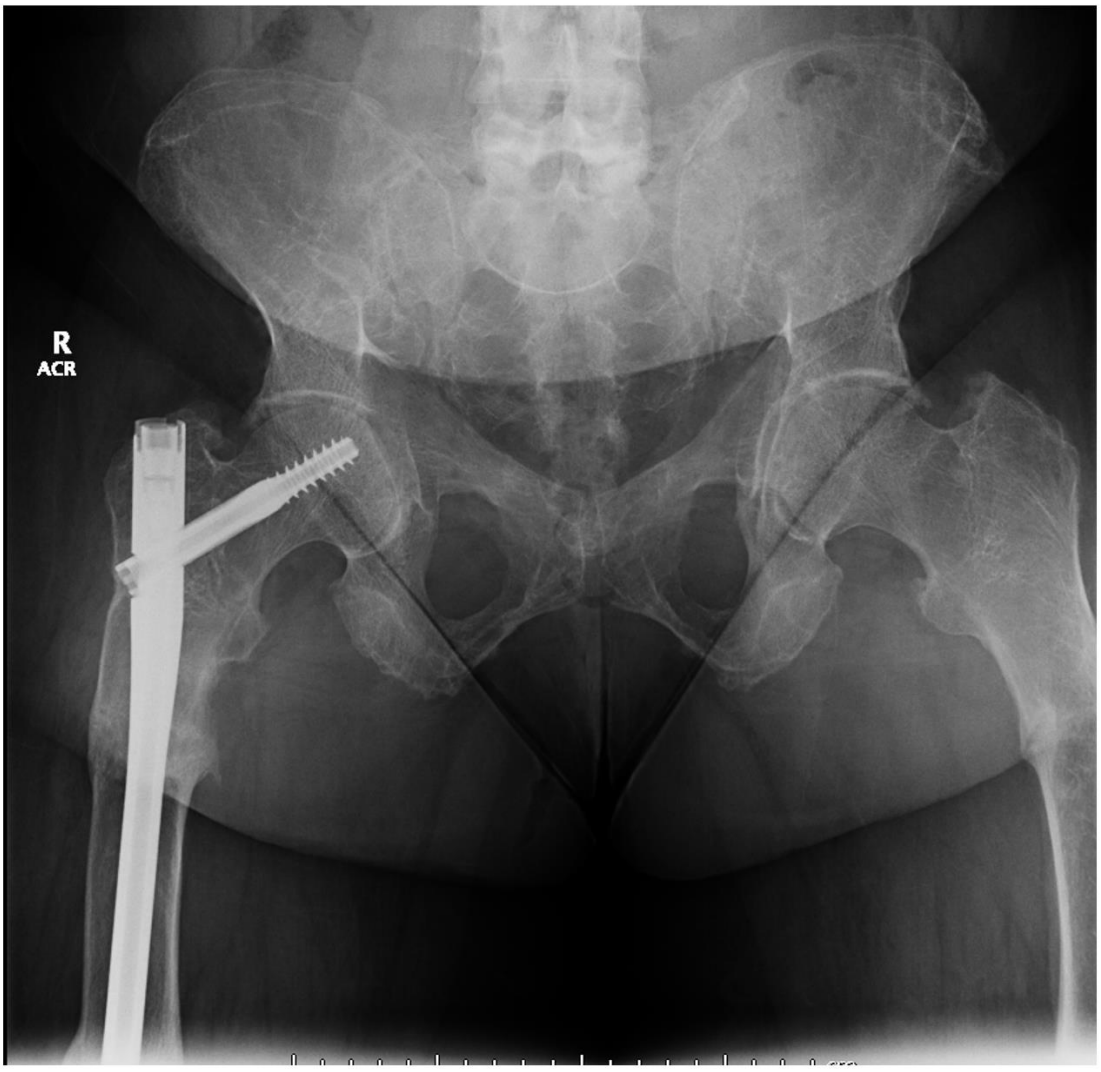

Figure 2. MB's pelvic X-Ray in 2014

We present a case of a 55 years old female with XLH that suffers from hypophosphatemia, hyperparathyroidism and non-healing left femoral fracture. This was further complicated by her inability to tolerate phosphate-calcitriol treatment. Her three children and granddaughter also have XLH.

Our burosumab application for her and her children has been approved. We believe that this is the first time burosumab has been approved for use in adult XLH patients in Australia. We aim that we will be able to report on her improvements at the time of ESA-SRB-ANZBMS 2021.

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Atypical femur fracture in a case of a young female with short stature and very high bone density

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Background: Pycnodysostosis is a rare, autosomal recessive disorder caused by loss of function mutation in the cathepsin K (*CTSK*) gene[1-2]. Typical features of this sclerosing bone dysplasia include short stature, skull and facial abnormalities and recurrent minimal trauma fractures with supra-normal bone mineral density and poor bone healing.

Case Presentation: A 24-year-old Iranian female, born to consanguineous parents, presented with an atypical femur fracture (AFF). A history of recurrent fractures was reported since the age of 7 involving bilateral tibia, humeral, metacarpal and metatarsal bones. Classical features of pycnodysostosis including short stature (150cm), and typical craniofacial and dental abnormalities were noted. A very high bone density on DXA further supported the diagnosis (femoral neck Z-score +7.0, lumbar spine Z-score +2.9).

Pycnodysostosis is a disease of dysfunctional Cathepsin K, an enzyme produced by osteoclasts and involved in degradation of bone matrix proteins[3]. Osteoclast dysfunction in this condition leads to reduced bone resorption, hardening of bone with micro-stress accumulation and fractures.

Early detection and diagnosis are beneficial as growth hormone treatment may normalise skeletal proportions and skeletal height if commenced early[4]. Management is otherwise supportive with environmental and occupational modifications, dental reviews and referral to medical specialists for various complications.

The occurrence of AFF in this condition sheds light on its pathophysiology in patients with osteoporosis on long-term bisphosphonates[5]. Our patient denied a family history of fracture and genetic testing of this patient is currently underway.

Conclusion: We describe a rare case of pycnodysostosis with multiple skeletal manifestations. Management is largely supportive, but early detection and referral is beneficial.

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Changes in bone turnover markers in adolescents with gastroesophageal reflux treated with lansoprazole

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Publish consent withheld

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Two cases with heterozygous *ENPP1* mutation presenting different skeletal manifestations

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Concurrent atypical femoral fracture and primary hyperparathyroidism. A clinical conundrum

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A 68-year-old Vietnamese woman sustained a right sided atypical femoral fracture (AFF), along with left metacarpal and distal radius fractures, following a fall from standing height. She was taking risedronate weekly for 6 years with prior vertebral insufficiency fractures. She had well-controlled hypertension, on perindopril.

Lumbar spine and femoral neck T-scores 11 years ago were -3.47 and -3.15, respectively. Six years ago, lumbar spine and femoral neck T-scores were -3.15 and -3.17, respectively.

Biochemical investigations (table 1) were consistent with primary hyperparathyroidism (PHPT). Despite prolonged antiresorptive therapy, bone turnover markers were in the upper range of normal in the setting of recent fracture and PHPT. No prior calcium testing was available.

Risedronate was ceased, and vitamin D supplementation commenced. An intramedullary nail was inserted in the AFF site, with an uneventful post-operative course. No concerning features of the contralateral femur were found on plain films and bone scintigraphy. The left metacarpal and left distal radius fractures were managed non-operatively.

Neck ultrasonography and parathyroid technetium-99m sestamibi demonstrated left inferior parathyroid adenoma. She underwent a minimally invasive parathyroidectomy soon after her femoral intramedullary nail insertion, with normalisation of serum calcium. Histology demonstrated a parathyroid adenoma.

This patient has concurrent issues of fragility fractures, bisphosphonate-associated AFF, and PHPT, posing a dilemma with future management. Bone turnover is high in PHPT and low in AFF, and to our knowledge there is only one case report of coexistence of both these conditions. The unexpected finding of PHPT in this patient on long term bisphosphonates highlights the importance of conducting a secondary screen for potentially reversible causes of osteoporosis at diagnosis and where treatment failure occurs. The increased cortical porosity found in PHPT may have increased the risk for developing an AFF. The incidence of AFFs in patients receiving antiresorptive therapies for PHPT is unknown.

Table 1. Biochemical Investigations

Test	Value	Reference range
eGFR	>90 ml/min	
Creatinine	42 umol/L	(40-90)
Corrected calcium	2.81 mmol/L	(2.15-2.65)
Phosphate	0.52 mmol/L	(0.75-1.50)
Albumin	35 g/L	(34-47)
25-hydroxyvitamin D	40 nmol/L	>50
Parathyroid hormone	11.7 pmol/L	(2.0-8.5)
Alkaline phosphatase	66 U/L	(30-110)
Bone specific alkaline phosphatase	23.6 ug/L	(3.8-22.6)
C-telopeptide	738 ng/L	(50-800)
P1NP	82 ug/L	(15-90)
24-hour urine (1630 mL)	Creatinine: 3.3 mmol/24h	(6.0-25.0)
	Calcium 3.3 mmol/24h	(2.0-7.5)
TSH	1.21 mIU/L	(0.5-4.0)
Calcium/creatinine clearance ratio	0.0127	>0.01 excludes FHH
PTH 10 days post parathyroidectomy	4.2 pmol/L	(2.0-8.5)
Corrected Calcium 10 days post parathyroidectomy	2.53 mmol/L	(2.15-2.65)

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Association of hypoparathyroidism with congenital bone abnormalities

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Background: Hypoparathyroidism is usually characterized by hypocalcaemia and a low or inappropriately normal level of parathyroid hormone. Hypoparathyroidism can be due to several underlying pathologies. Anterior neck surgery is the most common cause of acquired hypoparathyroidism, followed by other acquired causes such as autoimmunity, infiltration or radiation. Congenital or familial hypoparathyroidism may be caused by a number of established genetic defects. This case report describes a case of familial hypoparathyroidism with an autosomal dominant pattern of inheritance in a patient who has congenital bone abnormalities and was previously thought to have autoimmune hypoparathyroidism.

Case: A 43 year old female attends clinic for monitoring of hypoparathyroidism. Autoimmune hypoparathyroidism is listed as the cause across several health services over a period of 15 years including 2 pregnancies. At diagnosis corrected calcium was low at 2.02 mmol/L (reference range 2.1-2.6 mmol/L), phosphate elevated at 1.84 mmol/L (reference range 0.8-1.4 mmol/L) and PTH inappropriately low-normal at 1.6 pmol/L (reference range 1.6-6.9 pmol/L). Her medical history includes congenital absence of thumbs bilaterally, left ulnar hemimelia, seronegative rheumatoid arthritis and a pacemaker for tachy-brady syndrome. She is a non-smoker, drinks alcohol rarely and has two biologic children. Her medications are calcitriol 0.25mcg daily, calcium carbonate 1200mg daily, salazopyrin and sotalol, aiming for a serum calcium in the low-normal range. Following a detailed history, the patient revealed her sister has hypoparathyroidism and her deceased father had hypocalcaemia but declined PTH testing. On review of her children's biochemistry, it was found one had neonatal biochemistry (ionized calcium and PTH) consistent with hypoparathyroidism. The patient was referred to a genetics clinic and genetic testing is being performed.

Conclusion: We report the first case of hypoparathyroidism associated with congenital absence of ulna and thumbs, the underlying cause remains to be elucidated.

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Novel use of Combination BRAF and MEK inhibitor therapy for the Treatment of Diffuse Skeletal Erdheim-Chester Disease

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Background: Erdheim-Chester Disease (ECD) is a rare non-Langerhan's histiocytic disorder. ECD usually manifests as sclerosis of the long bones. Multi-organ involvement is common including pituitary infiltration, retroperitoneal fibrosis, and pericardial disease [1]. In more than 50% of ECD cases, ECD histiocytes are known to harbour the BRAF-V600E mutation but the precise pathophysiology is unclear [2]. Targeted therapies have shown efficacy in BRAF-V600-mutant ECD [3].

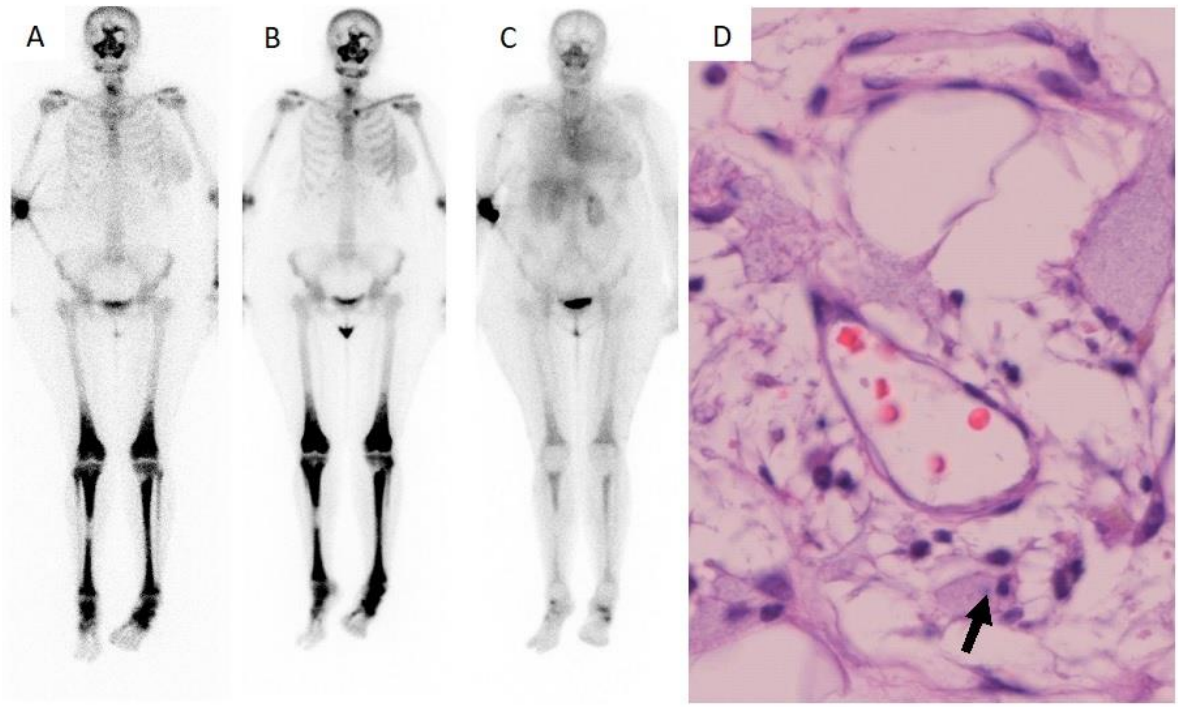
Case Presentation: We describe a case of diffuse skeletal ECD in a 63-year old female who presented with pain in the low limbs on the background recently diagnosed breast cancer. A staging bone scan for skeletal metastases displayed increased 99mTc activity bilaterally in the long bones of the upper and lower limbs (Figure 1A). Bone biopsy of the proximal tibia demonstrated lipid-laden, foamy histiocytes (see arrow, Figure 1D) with associated fibrosis and sclerosis, supporting a diagnosis of ECD.

BRAF codon 600 sequencing demonstrated a positive V600E mutation, a characteristic finding in BRAF mutant histiocytes. Extraskeletal involvement was excluded by FDG-PET and targeted imaging modalities.

ECD histiocytes are known to upregulate RANKL and induce local increase in osteoclast-mediated bone resorption. Hence, a RANKL inhibitor denosumab was administered at 120 mg monthly. However no demonstrable change was seen on 99mTc-bone scan after 3 months of treatment (Figure 1B) and her pain persisted.

Given BRAF-V600E mutation positivity of our patient's ECD, a novel combined treatment with a BRAF and MEK-inhibitor, dabrafenib and trametinib, was instituted. Pain improved within weeks and complete resolution in disease activity was demonstrated on serial bone scan after 6 months (Figure 1C).

Conclusion: This is the first case to describe the novel use of a BRAF and MEK inhibitor in the successful treatment and resolution of ECD confined to the skeleton. Treatment for ECD should be individualized based on organ involvement and molecular mutations.



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