



# PLENARY ABSTRACTS

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## **METABOLIC SENSING BY THE BRAIN: NEURONS THAT DO MORE THAN JUST SENSE GLUCOSE**

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The brain maintains the body's energy homeostasis by balancing energy intake, expenditure and storage. Specialized metabolic sensor neurons in the brain receive and integrate afferent neural and metabolic signals conveying information about the energy status of the body. These neurons are located in brain areas such as the hypothalamus, locus coeruleus, basal ganglia, limbic system and solitary nucleus, all of which are involved in homeostatic functions. Because glucose is a primary metabolic fuel, metabolic sensing neurons have evolved mechanisms to utilize glucose as a signaling molecule to control the rate of cell firing and neurotransmitter release. There are two types of glucosensing neurons that either increase (*glucose excited; GE*) or decrease (*glucose inhibited; GI*) their firing rate as brain glucose levels rise. Little is known about the mechanism by which GI neurons sense glucose. However, GE neurons appear to function much like the pancreatic  $\beta$ -cell where ATP generated by hexokinase-mediated glycolysis regulates the activity of an ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channel. The  $K_{ATP}$  channel is composed of 4 pore-forming units and 4 sulfonylurea binding sites. The presence of glucokinase may be the defining feature of a GE neuron where it acts as the gatekeeper for the glycolytic production of ATP. NPY and POMC neurons in the hypothalamic arcuate nucleus are critical components of the energy homeostasis pathways in the brain. Both have a glucosensing function and express Kir6.2, sulfonylurea receptors and glucokinase, as well as leptin and insulin receptors. In such neurons, leptin, insulin and long chain fatty acids act on the  $K_{ATP}$  channel to oppose the actions of glucose. These neurons also receive visceral neural and intrinsic neuropeptide and transmitter inputs. Thus, metabolism-related signals can summate upon the  $K_{ATP}$  channel to alter membrane potential, neuronal firing rate and peptide/transmitter release. The outputs of these neurons are integral components of effector systems which regulate energy homeostasis. Thus, arcuate NPY and POMC neurons are likely prototypes of this important class of sensor-integrator-effector neurons.

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## **CELLULOMICS OF THE MAMMALIAN OVARIAN FOLLICLE: EPIGENETIC DETERMINANTS OF RECRUITMENT AND SELECTION**

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A complex array of cellular interactions between the oocyte and somatic ovarian cells underlies the progressive nature of folliculogenesis. Cellular behaviors (cellulomics) are altered during the course of folliculogenesis and for the granulosa cell compartment these behaviors include proliferation, motility, apoptosis, and differentiation (luteinization). While mapping the course of gene and protein expression patterns has been instructive in revealing the changing landscape of intraovarian growth factors such as TGF $\beta$  family members (GDF9a, BMP-15), much remains to be understood with respect to cellular responses at critical stages of folliculogenesis (Elvin et al. 1999, McNatty et al. 1999). Central to an integrated view of regulatory interactions between the oocyte and granulosa will be definition of the cellular mechanisms that mediate intercellular communication at critical transitions during follicle development. We have proposed a model in which oscillatory signaling elicited by follicle stimulating hormone (FSH) modulates the availability of paracrine growth factors due to cytoskeletal remodeling in granulosa cell transzonal projections (TZPs; Albertini et al. 2001). Evidence supporting this model will be discussed with respect to (1) transcytotic potential of granulosa during the transition from pre-antral to antral follicles, (2) effects of FSH on cell-cell, cell-matrix interactions and the granulosa cell cytoskeleton, and (3) the phenotypic characterization of granulosa cell behaviors in mice carrying targeted deletions of GDF9a, connexin 37, and FSH- $\beta$ . From this analysis, it will be proposed that granulosa cell dynamics (cellulomics) are antagonistically modulated by oocyte factors and FSH, providing a conceptual framework for future studies into the process of ovarian folliculogenesis.

## NEUROENDOCRINE RESPONSES TO CRITICAL ILLNESS

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A severe illness which requires intensive care is characterized by lean tissue wasting despite feeding. This serious problem often persists even after the underlying disease has resolved and perpetuates intensive care dependency. A neuroendocrine dysfunction selectively in the chronic phase of illness was recently found to play a role. The initial neuroendocrine response to severe illness consists primarily of an activated anterior pituitary function while peripheral anabolic pathways are inactivated. This acute stress response provides metabolic substrates and host defence necessary for survival and thus has been considered adaptive and beneficial. Persistence of this acute stress response throughout the course of critical illness was hitherto assumed. This assumption has now been invalidated. Indeed, a uniformly reduced pulsatile secretion of at least TSH, LH, PRL and GH was observed in protracted critical illness, whereby impaired function of target organs. The reason for this is a hypothalamic rather than pituitary problem, as administration of relevant releasing factors evoked immediate and pronounced pituitary hormone release. A reduced availability of TRH, LHRH, one of the endogenous ligands of the GHRP receptor (such as the recently discovered ghrelin) and, in very long-stay critically ill men also of GHRH, is involved. This hypothesis was further explored by investigating the effects of continuous IV infusion of GHRH, GHRP, TRH and their combinations for several days and recently also the additional effects of IV LHRH pulses. Pulsatile secretion of GH, TSH, PRL and LH was re-amplified by relevant combinations of releasing factors which also substantially increased circulating levels of IGF-I, GH-dependent binding proteins, T4, T3 and testosterone. Active feedback inhibition loops prevented overstimulation of target organs. Metabolic improvement was most pronounced when GH-secretagogues, TRH and LHRH were administered together whereas the effect of single hormone treatment was minor. This new concept of reduced stimulation of pituitary function in protracted critical illness opens new therapeutic perspectives to reverse the paradoxical 'wasting syndrome' and intensive care-dependency. Treatment with releasing factors, as opposed to peripherally active hormones such as thyroid hormone, GH, IGF-I or anabolic steroids enables the body to adjust peripheral hormonal metabolism and activity according to the needs determined by the disease process. This is an important safety aspect for an endocrine treatment at a time when it is virtually impossible to determine 'normal' or 'optimal' circulating levels of different peripherally active hormones. Whether this novel endocrine strategy will also enhance clinical recovery from critical illness remains to be explored.

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## GENOMES, BIOINFORMATICS AND THE FUTURE OF SCIENCE

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The recent completion of the human genome, together with completed genomes of many bacteria, yeast, fruit fly and the worm *C. elegans* has provided an enormous amount of sequence information that provides a starting point for novel biological studies. In addition, scientists have developed a large number of high-throughput methods of experimentation including microarrays, proteomics and structural studies that analyse the actions of thousands of genes simultaneously. How does one make sense of so much data? One answer is the new discipline of Bioinformatics which is the intersection of information technologies and applied mathematics with molecular biology and genetics. It provides the tools to collect, store and analyse this new information. In particular, Bioinformatics provides one of the most important techniques for the scientific and commercial exploitation of DNA sequence information.

This lecture will review a number of these developments including findings from a number of genome projects, particularly the human genome project. The role of Bioinformatics in analysis of the genome will be illustrated together with a discussion of some of the significant problems still to be resolved. A number of "post-genomic" technologies such as microarrays and high throughput structural biology will also be discussed.

Much of the genomic information is in the public domain and available to scientists in Australia. Those countries able to exploit this information will create many opportunities for important discoveries and innovation in the biomedical, agricultural and other areas and the consequent commercial bonanza. Yet many of the technologies required to analyse such information are extremely expensive and require specialised apparatus and highly skilled operators. The low amount of funding allocated to science in this country creates significant problems for those wishing to be internationally competitive. Ways in which Australia may try to be part of this scientific revolution will be discussed.

## PTHrP-FROM ENDOCRINOLOGY TO INTRACRINOLOGY

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Parathyroid hormone – related protein (PTHrP) was discovered as a hormone responsible for the humoral hypercalcemia of malignancy through its PTH-like actions in promoting bone resorption and reducing calcium excretion. Produced commonly also by breast cancers, it may confer upon these cells a property suiting them to grow as metastases. This latter property determined largely by the influence of the bone microenvironment, and indeed a long term, prospective clinical study suggests that an important effect of PTHrP is to reduce breast cancer invasiveness and general metastatic capacity. The hormonal functions of PTHrP other than in cancer appear to be to regulate placental calcium transport, and possibly also to exert actions during lactation.

In its physiological functions – PTHrP has emerged as a multifunctional protein, important in skin, blood vessels, heart, breast and uterus. In addition it emerges that PTHrP is a nuclear/nucleolar protein in certain tissues and that this localization is cell cycle-regulated, mediated by the middle portion of the molecule, and that PTHrP is transported into the nucleus by the nuclear import receptor importin  $\beta$ . The nuclear functions are yet to be defined, but an intracrine role appears certain, in addition to the paracrine and endocrine roles of PTHrP.

## TREATMENT STRATEGIES OZ.DOC

### STRATEGIES FOR THE PREVENTION AND TREATMENT OF OSTEOPOROSIS

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In the past 10 years there have been significant advances in the field of osteoporosis, including the development of diagnostic techniques, a greater understanding of the pathogenesis of osteoporosis and the evolution of effective treatments. There are, however, large gaps in our ability to implement effective therapeutic strategies. Strategies may be based on a global approach where the intention is to shift the distribution of bone mineral density (BMD) in the whole population, for example by the promotion of exercise, smoking cessation or manipulating the dietary intake of calcium. However, evidence for the efficacy of such approaches is lacking, and their feasibility has never been tested. The alternative approach is the 'high risk' strategy whereby segments of the population most at risk are targeted for intervention, for example, mass population screening of women at the time of the menopause. There are many reasons why this is not feasible at present.

A particular problem with BMD is that despite its high specificity, its sensitivity (detection rate) for fracture outcomes is low over most reasonable assumptions. For this reason, treatment strategies need to take into account the risk factors that operate independently of BMD and thereby enhance the predictive value of the test. These factors include age, family history of hip fracture, prior fragility fracture, high rates of bone turnover, low body mass index and neuromuscular incompetence. The presence of such factors increases fracture risk over and above that which can be explained on the basis of BMD. Therefore, diagnostic thresholds differ from intervention thresholds. Intervention thresholds should be based on the absolute risk of the clinically significant outcomes.

## FORKHEAD GENES AND INSULIN ACTION: THE LIGHT AT THE END OF THE TUNNEL?

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The pleiotropic actions of insulin are mediated by a single receptor tyrosine kinase, which acts through insulin receptor substrates (IRSs) to stimulate phosphatidylinositol (PI) 3-kinase and a host of PI-dependent kinases. The presence of multiple insulin-regulated, PI-dependent kinases is consistent with the possibility that different pathways are required to regulate different biologic actions of insulin. Genetic evidence in the nematode *Caenorhabditis elegans* has suggested that transcription factors of the forkhead family are important for insulin receptor signaling. Forkhead proteins belonging to the *Foxo* (FKHR) subclass are phosphorylated in an insulin-responsive manner by PI-dependent kinases, such as Akt. Phosphorylation inhibits their transcriptional activity via nuclear exclusion. Using transgenic and knockout mice with gain- and loss-of-function mutations, we show that *Foxo* proteins are distal effectors of insulin action in three key target organs: liver, pancreatic beta cell and adipose tissue. Mice bearing a single null allele of *Foxo1* (*Foxo1*<sup>+/-</sup>) rescued insulin resistance in mice with impaired insulin signaling due to an insulin receptor heterozygous knockout (*Ir*<sup>+/-</sup>). Moreover, a gain-of-function mutation of *Fkhr* targeted to the liver of transgenic mice caused severe glucose intolerance. These data indicate that *Foxo1* haploinsufficiency restores insulin sensitivity in insulin-resistant mice, whereas over-expression of a constitutively active mutant *Foxo1* causes insulin resistance. *Foxo1* is the main member of this family of transcription factors in pancreatic beta cells. We show that a heterozygous *Foxo1* null mutation restores beta cell mass in *Irs2* knockout mice, due to increased proliferation and differentiation of pancreatic duct cells into beta cells. Finally, we demonstrate a key role for *Foxo1* in the process of adipocyte differentiation. To this end, we examined the effects of constitutively active and dominant negative *Foxo1* in three models of adipocyte differentiation: 3T3-F442A cells, mouse embryonic fibroblasts (MEF) and mice with targeted null mutations in insulin receptor (*Ir*) and *Foxo1* (*Ir/Foxo1*<sup>+/-</sup>). Transduction of 3T3-442A preadipocytes with adenovirus encoding constitutively active mutant *Foxo1* inhibited adipocyte differentiation and suppressed expression of key adipocyte-specific genes, such as PPAR gamma, GLUT4 and leptin. Conversely, a dominant negative *Foxo1* enhanced adipocyte differentiation of MEF, and restored the ability of MEF from *Ir* knockout mice to undergo adipocyte differentiation. The effects of loss-of-function mutations of *Foxo1* on adipocyte differentiation was examined in vivo. *Foxo1* haploinsufficiency reduced epididymal fat pad size in *Ir*<sup>+/-</sup> mice and was increased adipocyte number, suggesting that the *Foxo1* mutation favored the formation of smaller adipocytes. These data indicate that *Foxo1* activates genes that inhibit the differentiation process, and that *Foxo1* inhibition by insulin is required for the attainment of the fully differentiated adipocyte phenotype. *Foxo1* appears to act as a molecular linchpin between peripheral insulin action and pancreatic beta cell function, thus raising the possibility that molecular defects in *Foxo1* function underlie the genetic predisposition to type 2 diabetes.

## THE NUCLEAR RECEPTOR SUPERFAMILY: WHAT ELSE WILL THESE PROTEINS BE FOUND TO DO?

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The nuclear receptor superfamily consists of about fifty proteins and variants that are ligand-regulated transcription factors. The classical receptors for steroids were joined in our thinking by thyroid, vitamin D and retinoids. More recently, related proteins of this sub-family have been found to be the sensing proteins in liver that are responsible for controlling both phase 1 and phase 2 drug metabolism via regulation of enzyme levels for the major cytochrome P450 proteins. Now the list of regulated genes has reached the intestine, where the drug efflux pump, MDR1, is also regulated by these nuclear proteins. Pharmaceutical probes have elucidated amazing structural and functional aspects of these proteins. The talk will review the protein family, their physiologic and structural functionalities and the interactions among family members that presage important new drug-discovery paradigms for the future.

## MOLECULAR CHARACTERISATION OF GRANULOSA CELL TUMOURS OF THE OVARY

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Cancer of the ovary is the most common fatal gynaecological malignancy. The majority of ovarian cancers derive from the surface epithelium of the ovary being classified as either serous or mucinous cystadenocarcinomas by morphological criteria. ~10% of ovarian tumours are granulosa cell tumours (GCT). GCT share many features of normal granulosa cells (GC) in that they synthesise gonadal peptides including inhibin and Müllerian inhibitory hormone and the gonadal steroid oestradiol. Inhibin secretion has proven to be a clinically useful tumour marker. In our studies of ovarian tumours we have focused on the biology of GCT. The findings in the GCT have been contrasted with those from groups of normal premenopausal ovaries, as well as mucinous and serous cystadenocarcinomas. Several specific hypotheses have been examined: Firstly we have sought to define the relationship of GCT to GC by defining the patterns of expression of known GC genes in the GCT. Genes which have been examined include the receptors for FSH, LH and oestradiol as well as the inhibin subunits, aromatase SF-1, COX-2, cyclin D2, RII $\beta$  and sgk genes. The pattern of gene expression observed in the GCT would suggest a phenotype that is similar to that of the GC of a late preovulatory follicle. These studies are currently being extended to a wider pool of genes using macroarrays. We and others have postulated that this phenotype would be consistent with constitutive activation of the FSH signalling pathways; we have however been unable to find evidence of mutations in either the FSH receptor or the associated G $\alpha$  subunit genes. This may reflect activation of this signalling pathway downstream of the receptor complex. Thirdly we have examined the patterns of ER $\beta$  isoform expression in the GCT. The pattern of expression of steroid receptor coregulatory molecules that may be involved in mediating the estrogen response have also been determined. GCT have high levels of ER $\beta$  expression with prominent expression of SRC-1 and SRC-2 but not SRC-3. In murine models, deletion of the inhibin  $\alpha$ -subunit gene results in GCT formation whereas in humans, GCT synthesise and secrete biologically authentic inhibin. It has been postulated that this apparent contradiction between the experimental model and the clinical situation may reflect resistance of the GCT to inhibin. Activin/inhibin receptor subunit gene expression is unexpectedly heterogeneous in the GCT particularly the inhibin B specific subunit, p120. Finally we have sought to extend these studies to an *in vitro* model using two cell lines derived from human GCT. The status of the signalling pathways within these lines is currently being defined as are the responses to estrogen. The similarities between the GCT and GC of late preovulatory follicles suggests several mechanisms by which GCT may arise. It can be hypothesised that the pathogenesis of GCT involves mutations which cause: activation of a signalling pathway such as the FSH receptor effector pathway, failure of terminal differentiation/luteinization, inactivation of a suppressor signal such as inhibin and/or failure of the normal atretic/apoptotic fate of most GC.

## MECHANISMS OF FAILURE OF ANDROGEN ABLATION THERAPY FOR ADVANCED PROSTATE CANCER

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Consistent with both the development of the normal prostate gland and prostate tumorigenesis being dependent on testicular androgens, targeting the androgen signalling axis (ie androgen ablation therapy) remains the predominant treatment regime for patients with metastatic prostate cancer. While there is a very good initial response to androgen ablation, these treatments are essentially palliative. Recent evidence suggests that treatment failure may not result from a loss of androgen signalling, but rather the acquisition of genetic changes that lead to aberrant activation of the androgen signalling axis. The expression of the molecular target of androgens, the androgen receptor (AR), is maintained in virtually all advanced tumours, and amplification of and mutations in the AR gene have been detected in up to 30% and 50%, respectively of clinical specimens. In addition, it was recently determined that the AR could be activated in the absence of ligand by growth factors and cytokines.

A consistent finding is that androgen receptor (AR) gene mutations present in metastatic prostate cancer, and in human prostate cancer cell lines as well as xenograft and other animal models, result in decreased specificity of ligand-binding and inappropriate receptor activation by estrogens, progestins, adrenal androgens, glucocorticoids and/or AR antagonists. Since a significant proportion of missense mutations in the AR gene reported in prostate cancer collocate to the signature sequence and AF-2, two discrete regions of the ligand-binding domain critical for androgen signalling, we recently proposed that this collocation would identify additional regions of the AR important in receptor function. This approach led to the identification of a four amino acid region at the boundary of the hinge and ligand-binding domains of the receptor that forms half of a potential protein-protein binding site. We have used a combination of biochemical analysis and molecular modelling to explain altered biological activity for AR variants in each of these three regions. In addition, we have also determined that AR gene mutations identified in prostate cancer also collocate to areas in the DNA binding domain, the amino terminal transactivation domain and to the hinge region. In nearly every case, missense mutations in the AR gene identified in prostate cancer that collocate to discrete regions of the receptor contribute to altered androgen signalling, and provide a potential mechanism to explain the re-emergence of tumour growth during the course of hormone ablation therapies.

## ENDOCRINE COMPLICATIONS IN ADULTS TREATED FOR CANCER IN CHILDHOOD AND ADOLESCENCE

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Survivors of childhood cancer frequently develop disturbances of the endocrine system as a result either of their cancer diagnosis, or more commonly, as a consequence of curative therapy. Adult survivors may require both ongoing care and treatment for endocrine disorders acquired during childhood, as well as surveillance and testing for new disorders that may develop only many years after completion of cancer therapy. The following are the more prevalent endocrine disturbances seen in this population:

**Neuroendocrine.** Growth hormone deficiency (GHD) occurs commonly following irradiation (RT) of the hypothalamic-pituitary unit. Treatment with high doses of RT (>30 Gy) often results in GHD within 5 years of diagnosis, while in those treated with lower doses (18-24 Gy) GHD may not develop for >10 yrs post-treatment. Some of these adult survivors will benefit from treatment with GH to ameliorate the metabolic and psychosocial derangements associated with the Adult GHD syndrome. The benefits and safety of long-term GH therapy in these individuals have not yet been determined. Adult survivors exposed to >30 Gy RT remain at risk of developing deficiencies of TSH, LH/FSH, and ACTH over time.

**Thyroid.** RT to the neck is correlated with an elevated risk for hypo- and hyperthyroidism, as well as thyroid neoplasms, both benign and malignant. These disorders are more common in females treated with higher doses (>25-35 Gy) of RT and may not become evident for >20 yrs after treatment.

**Gonadal/reproduction.** While most females retain ovarian function following cancer therapy, a subset of female survivors (eg, treated with alkylating agents and/or pelvic RT) are at increased risk of entering a premature menopause (< age 40). Overt Leydig cell failure is uncommon but subtle Leydig cell dysfunction (normal-low testosterone, raised LH) is noted frequently, especially in survivors treated with intensive combination chemotherapy. Preliminary data suggest the latter may be at risk of disordered body composition and reduced bone mineral density. Optimum management of gonadal failure in young adult survivors remains to be established. Fertility is reduced in certain subgroups of childhood cancer survivors, especially in males treated with alkylating agents and/or scattered RT to the testes. In females, RT to the pelvis is associated with a greater incidence of spontaneous abortions, low birthweight and prematurity. For female survivors who are infertile, the role and safety of assisted reproductive techniques are largely unknown.

## CLINICAL IMPORTANCE OF ENDOCRINE THERAPY FOR BREAST CANCER.

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Traditionally endocrine manipulations have arbitrarily divided women with breast cancer into two broad groups, pre-menopausal and post-menopausal. Moreover the therapeutic approaches used have varied depending on whether women have had metastatic breast cancer, or early (curable) breast cancer. Endocrine manipulations were used almost exclusively for women with metastatic breast cancer until the 1970's when the first clinical trials reporting the effects of tamoxifen became widely available. For pre-menopausal women with metastatic breast cancer, traditional treatments included either a surgical, radiation induced or pharmacologic oophorectomy with a GnRH agonist. The oestrogen receptor content of the primary or secondary tumour is a strong predictor of tumour response and only women whose tumour expresses the oestrogen receptor should be offered hormonal therapy. Approximately 60% of this group of women (both pre-menopausal and post-menopausal) will respond to endocrine therapy. Other endocrine manipulations include the addition of tamoxifen, a progestational agent and more recently an aromatase inhibitor. These treatments are usually offered sequentially upon the progression of the underlying breast cancer. Clinical trials have recently demonstrated that the third generation aromatase inhibitors are more effective than progestational agents and are at least equally effective, and in some cases more effective, than tamoxifen. However the aromatase inhibitors are only active in post-menopausal women. Adjuvant therapy has recently been identified as having a significant favourable impact on survival in women with early, curable breast cancer. Early trials with ovarian ablation in pre-menopausal women did not demonstrate an improvement in overall survival. Recent meta-analyses of all relevant trials in this group have demonstrated that ovarian ablation does appear to improve survival. Other trials have compared ovarian ablation, mostly with the use of chemical ovarian ablation against adjuvant chemotherapy. Preliminary data suggests that ovarian ablation is as effective as chemotherapy. However, more data is required as some of these trials have used older types of chemotherapy or chemotherapy which is believed to be administered sub-optimally (either reduced dose or a less effective schedule) than newer forms of chemotherapy. Importantly the use of adjuvant tamoxifen has been conclusively demonstrated to improve overall survival in both pre-menopausal women and post-menopausal women. Tamoxifen must be administered for at least five years to have this benefit and further trials are ongoing to address the question of whether more than five years of tamoxifen will provide further improvements in survival. Current clinical trials are addressing the question of the use of the aromatase inhibitors as adjuvant hormonal agents in post-menopausal women. Some trials have been completed but most are ongoing and as yet no data are available. Other interesting areas to be aware of in the future include the development of the third generation selective oestrogen receptor modulators and the new oestrogen receptor down regulator, fulvestrant (Faslodex®). One can therefore see the enormous benefit gained from endocrine hormone treatment of women with breast cancer, with future clinical trials both further refining the population of women benefiting as well as the development of new treatments.

## THE ROLE OF HYALURONAN IN OVULATION, FERTILISATION AND EMBRYOGENESIS.

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Hyaluronan (HA) is a large polysaccharide in the extracellular matrix (ECM), which consists of repeating disaccharide units of D-glucuronic acid and N-acetyl-glucosamine. Despite its relatively simple composition hyaluronan fulfils several distinct molecular functions that contribute to both the structural and physiological characteristics of tissues and to cell behaviour during reproduction and embryogenesis. Synthesis of hyaluronan and its organisation into an ECM are processes involved in successful ovulation and fertilization in most mammals including human.

The synthesis and accumulation of hyaluronan contributes to the above processes at several levels. Firstly, HA contributes directly to tissue homeostasis and biomechanics due to its unique biophysical properties, a function which relates directly to the expansion of the preovulatory cell-oocyte complex (COC) where HA attracts water, causing an increase in the volumetric domain of the intercellular spaces shortly before ovulation. The hydration homeostasis abilities of HA are also imperative during tissue formation or remodelling where the HA matrix provides a hydrated, fluid pericellular environment in which assembly of other matrix components and presentation of growth and differentiation factors can readily occur without interference from the highly structured fibrous matrix usually found in fully differentiated tissues.

Secondly, interactions of HA with link proteins and proteoglycans are of fundamental importance to the structural integrity of extracellular and pericellular matrices of reproductive cells and rapidly proliferating tissues. In the cumulus cells and COC matrix several other proteins interact with HA to form a stable environment to facilitate the extrusion of the oocyte at ovulation and to act as a selective barrier that excludes dysfunctional sperm, while in embryogenesis the binding of proteoglycans to pericellular HA may transform the pericellular matrix from conducive to inhibitory for cell migration and proliferation.

Thirdly, interactions with cell surface hyaluronan receptors mediate significant influences on cell behaviour such as migration and proliferation. In reproduction HA receptors such as CD44 appear to be essential in the binding of HA to the cumulus and in the stabilisation of the COC. While in morphogenesis of embryonic organs the receptors perform many functions such as the controlled removal of HA enabling cell differentiation.

An overview of the role of hyaluronan synthesis, degradation and receptors during ovulation, fertilization and embryogenesis will be presented with the aim of adding a new perspective to the role of hyaluronan in these processes.

## FIBRONECTIN AND ITS PROTEOLYTIC FRAGMENTS: DIVERSE ROLES IN MAMMARY GLAND INVOLUTION AND CARTILAGE DEGRADATION.

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The maintenance of tissue architecture is a dynamic process, involving a reciprocal flow of information between the cell and its surrounding matrix. Fibronectin (Fn) is an extracellular matrix glycoprotein that is found in most body fluids and soft connective tissues, with elevated expression often associated with tissues undergoing remodelling or repair. Fn plays an essential role in embryonic development, and influences a diverse range of cellular processes including migration, differentiation, proliferation and apoptosis.

Fn is a disulphide-bonded dimer, with various isoforms resulting from alternative splicing of the primary transcript. Distinct functional domains have been identified by studying fragments of fibronectin (Fn-fs) in vitro. Cellular interaction with Fn is via integrins, with a well-documented integrin binding site mapped to the central cell-binding domain (Fn-f 120). Focal adhesions are augmented by interactions between Fn and cell surface heparan sulphate proteoglycan or tissue transglutaminase. Fibronectin also comprises regions that bind other matrix molecules, important in the formation of a provisional matrix during wound healing.

Deposition of Fn in tissues leads to irreversible unfolding of the molecule to form a matrix of interconnected fibrils. Certain functional domains of Fn that are buried in the soluble molecule become fully exposed upon surface-adsorption or binding to other matrix molecules. Cells migrating on Fn bend and stretch the fibrils, such that cryptic domains may be transiently available for cellular interaction. Proteolytic fragmentation of fibronectin also leads to the release of cryptic domains. A common finding in the literature is that Fn-fs have biological properties distinct from those of the full-length molecule. This will be discussed, with reference to two examples in which Fn-fs regulate metalloproteinase activity, during mammary gland involution and the breakdown of articular cartilage in joint disease.

Post-lactational mammary gland involution involves remodelling of the mammary gland from a tissue rich in alveolar epithelium to a tissue composed of sparse ductal-like epithelium. Studies have shown that peak expression of Fn and Fn-fs is coincident with peak mammary epithelial cell (MEC) death during involution. Treatment with Fn-f 120 induces MEC apoptosis and the expression of certain matrix metalloproteinases (MMPs)<sup>1</sup>. MEC cells plated on basement membrane matrix will switch from forming alveolar-like structures to duct-like structures when Fn-f 120 is added to the cultures. Since this switch can be inhibited by the addition of an MMP inhibitor, this illustrates the influence that Fn-fs can have on MMP-mediated matrix remodelling.

Our focus is on the N-terminal Fn-fs, and on the role these fragments play in the degradation of articular cartilage. The primary function of cartilage as a weight bearing tissue is dependent upon its unique matrix, composed of two key molecules, collagen II and aggrecan. Loss of aggrecan from cartilage is one of the early markers of osteoarthritis, and has been attributed to proteolysis by aggrecanases (ADAMTS metalloproteinases) and MMPs. In the later stages of osteoarthritis, collagen II is also subject to proteolysis by MMPs, leading to irreversible tissue damage. Fn levels increase in early arthritis, possibly as part of a tissue repair response. However, it is likely that proteolytic generation of Fn-fs exacerbates arthritis, since Fn-fs (unlike Fn) upregulate cartilage catabolism. We have observed that treatment of cartilage with N-terminal Fn-f 45 (collagen binding fragment) upregulates aggrecanase-mediated breakdown of aggrecan, as well as the synthesis of MMP-13, the collagenase most associated with the degradation of collagen II. Understanding the mechanism by which Fn-fs signal catabolic pathways will be essential for the design of therapies aimed at limiting proteolytic damage to cartilage matrix.

(1) Schedin, P, Strange, R, Mitrenga, T, Wolfe, P, Kaeck, M (2000) *J Cell Science* 113:795-806



## INVASIVE PHENOTYPES ASSOCIATED WITH HORMONE-INDEPENDENCE IN BREAST AND PROSTATE CANCER CELL LINES: IMPLICATION OF THE EPITHELIO-MESENCHYMAL TRANSITION

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One of the key events for carcinoma metastasis is the dissociation of individual cells from the primary tumour mass. This involves deregulation of the cell adhesive proteins, and upregulation of integrins used for cell:substratum interactions. Although degradation of the basement membrane is also critical for the dissemination of these cells, microinvasive loss of the basement membrane from the periphery of the tumour in breast carcinoma does not automatically confer metastatic spread, suggesting that additional events are required. One possible mechanism is the epithelio-mesenchymal transition (EMT), whereby epithelial cells, and possibly their carcinomatous derivatives, will lose epithelial characteristics and adopt a motile, mesenchymal phenotype. The EMT is a major mechanism for relocation of epithelial cells in the developing embryo, and has been invoked previously as a carcinoma metastasis mechanism. Our interest in this possibility was raised by observations that invasive and metastatic human breast cancer cell lines overexpress the mesenchymal intermediate filament protein vimentin in preference to the epithelial keratins. They also express the mesenchymal-associated membrane-type 1-MMP (MT1-MMP), overexpress TIMP-2, and are capable of activating the so-called 72 kDa type IV collagenase (MMP-2). All estrogen-responsive cell lines showed epithelial traits, were poorly motile, non-invasive, and poorly metastatic in immunocompromised hosts, suggesting that oestrogen-dependent metastases occur through other mechanisms. Alternatively, these metastases may form by re-differentiation of the disseminated cells, as is often the case in the embryo.

Recently we extended these studies to human prostate cancer cell lines, and found a striking parallel. Androgen-dependent LNCaP cells were poorly motile, barely invasive, and did not express MT1-MMP or activate MMP-2. In contrast, the three androgen-independent lines tested (DU-145, TSU-Pr1, PC-3) showed abundant vimentin expression, much higher levels of motility and invasiveness, and MT1-MMP expression / MMP-2 activation. Furthermore, the C4-2B LNCaP subline which has become hormone-refractory and metastatic to bone showed partial vimentin expression and increased invasiveness, but did not activate MMP-2.

These data provide additional support to the likelihood that EMT mechanisms are instrumental in the dissemination of hormone-refractory breast and prostate cancer, and may also participate temporarily in the formation of hormone receptor positive metastases. Gene array analyses of these cell lines is ongoing in a number of laboratories including our own, with the goal of more comprehensive characterisation of the gene expression profiles associated with these phenotypes.

## REGULATION OF A PUTATIVE METASTASIS-PROMOTING PROTEOGLYCAN IN PROSTATE STROMA BY CANCER CELL PRODUCTION OF TRANSFORMING GROWTH FACTOR BETA-1

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Versican is a large chondroitin sulphate proteoglycan found in trace amounts in periglandular ECM of the mature prostate gland. The function of this proteoglycan in the normal prostate is unclear, but the molecule is known to possess growth factor-like activity, and to be an important regulator of cell motility during the development of neural tissues. We have previously reported (1) that versican-rich peritumoral stroma is predictive of relapse after surgery for early stage prostate cancers, thereby implicating versican expression with local spread and metastasis of prostate cancer cells prior to surgery.

Immunohistochemical staining and RT-PCR studies suggest that versican is a product of prostatic stromal cells and not the cancer cells (1, 2). Conditioned medium from LNCaP, PC3 and DU145 prostate cancer cell lines induced increased secretion of versican by cultured prostatic fibroblasts. Collectively, these data suggest that prostate cancer cells produce factors, which regulate stromal production of versican. To test this hypothesis, the effect of neutralising antibodies specific for several prostatic growth factors on stromal versican induction by prostate cancer cells was examined. Only antibody to transforming growth factor beta-1 (TGFβ1) inhibited stromal versican production, suggesting that this cytokine was critical for regulating peritumoral versican expression. Using the normal prostate gland of the guinea pig, periglandular expression of versican was shown to be down-regulated during pubertal development and by administration of dihydrotestosterone (DHT) to castrated animals. This reduction in versican expression may result from a down-regulation of TGFβ1 by circulating androgens, but this implication has not been examined at present.

To further examine whether versican is important for prostate cancer cell metastasis, versican-containing culture medium from prostatic fibroblasts was tested for its ability to block attachment of LNCaP, PC3 and DU145 prostate cancer cells to fibronectin-coated or laminin-coated surfaces. Versican-containing culture medium inhibited cell attachment to fibronectin by 60%, whereas attachment to laminin was unaffected. These studies suggest that versican may promote cancer cell metastasis through the prostate stroma via stromal-epithelial interaction. The finding that TGFβ1 is a critical regulator of epithelial cell-induced production of stromal versican suggests both are relevant targets for controlling the spread of prostate cancer cells.

## LIPID-INDUCED INSULIN RESISTANCE IN MUSCLE

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It is well documented that accumulation of triglyceride (TG) in human and animal muscle tissue is strongly associated with a reduction in the ability of insulin to regulate glucose metabolism in this tissue (insulin resistance). How lipid metabolism interferes with insulin action is not well understood. Because TGs are storage products, it is unlikely that they directly interfere with insulin signalling pathways. However, there is increasing evidence for intracellular lipid metabolites having specific and direct effects on insulin action and glucose metabolism. The first step in fatty acid (FA) metabolism (oxidation or storage) is activation to the long chain fatty acyl CoA (LCACoA). LCACoAs can then be transferred to the mitochondria for oxidation or converted to diacylglycerols (DAG) and thence TG. We have recently demonstrated that changes in the intracellular concentration of LCACoA are correlated with changes in insulin action in muscle from dietary and genetic models of insulin resistance. LCACoA concentration can directly influence glucose metabolism by inhibiting hexokinase activity in muscle. Serine phosphorylation and inhibition of proteins of the insulin signalling pathway by PKCs has been proposed as a mechanism for lipid-induced insulin resistance. DAGs can activate the novel protein kinase C (PKC) isozymes theta and epsilon and both DAG and activation of these PKCs are increased in lipid-induced insulin resistance. In addition, different fatty acids have different effects on insulin signalling. Incubation with palmitate increased ceramide concentrations and decreased PKB activation while unsaturated fatty acids reduced IRS-1 phosphorylation in a murine muscle cell line. Longer term effects of lipids on glucose metabolism and insulin action may involve regulation of gene expression via transcription factors (such as the PPARs) which are activated by fatty acids. Thus it would seem that there are multiple mechanisms linking lipid accumulation with cellular insulin resistance. Elucidation of these mechanisms may provide new dietary and drug strategies for the treatment of insulin resistance in diabetes.

## INSULIN RESISTANCE IN HUMAN ADIPOSE TISSUE

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Obesity and pregnancy are recognised as states of insulin resistance. Adipose tissue is important in whole body glucose homeostasis as shown in mice with adipose tissue specific reduction of GLUT4 displaying insulin resistance in vivo in muscle and liver. [1] We hypothesise that in man, abnormal glucose uptake occurs in adipose tissue in states of insulin resistance. We have investigated basal and insulin stimulated glucose uptake in adipose tissue from two depots in obese and pregnant subjects. In addition we have assessed the effect of TNF-alpha and dexamethasone, compounds known to impair insulin sensitivity. Adipose tissue was obtained at abdominal surgery or caesarean section and basal and insulin stimulated glucose uptake was measured in explants using [<sup>3</sup>H]-2-deoxyglucose.

Insulin stimulated glucose uptake is impaired at body mass index (BMI) >25. Central adiposity was associated with impaired insulin stimulated glucose uptake in omental tissue and there was a trend for reduced glucose uptake with age. Basal glucose uptake (non-insulin-mediated) also declined with increasing BMI. In pregnancy insulin stimulated glucose uptake was comparable with prepregnancy BMI matched nonpregnant women. Corticosteroids reduced basal and insulin stimulated glucose uptake in omental but not subcutaneous fat. TNF-alpha effects were BMI-dependent with a trend to increased basal glucose uptake in lean omental tissue and decreased insulin stimulated glucose uptake in lean and obese subcutaneous tissue.

These findings demonstrate: 1) BMI, anatomical depot, body fat distribution and age all influence insulin action and glucose disposal in human adipose tissue. 2) Non-insulin-mediated glucose uptake has an important role in glucose homeostasis. 3) Pregnancy per se may not influence insulin action in adipose tissue.

## INSULIN REGULATION OF GLUCOSE TRANSPORT IN ADIPOCYTES

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Insulin stimulates glucose transport in muscle and fat cells by triggering the translocation of the glucose transporter GLUT4 from intracellular vesicles to the cell surface. Two areas related to this process will be discussed:

**IRS1 localisation.** The insulin signalling pathways that regulate glucose transport have been extensively studied. IRS1, a direct substrate of the insulin receptor tyrosine kinase, plays a pivotal role in insulin action. Tyrosyl phosphorylated IRS1 acts as a scaffold for signalling molecules such as PI 3' kinase. PI 3' kinase regulates the activity of Akt which has been shown to play a key role in insulin-stimulated glucose transport. We have previously suggested that IRS1 is attached to the cytoskeleton and that this localisation might play a key role in IRS1 function. Agonists that disrupt this localisation also stimulate Ser/Thr phosphorylation of IRS1 and impair insulin action. We have now shown using high resolution EM that IRS1 is localised to microtubules and intermediate filaments in adipocytes and CHO cells. Furthermore, by making a series of truncation mutants we have identified the central domain of IRS1 adjacent to the SAIN domain as playing a critical role in the cytoskeletal anchoring of IRS1.

**2. Role of calcium.** Both the calcium chelator, BAPTA-AM, and the calmodulin antagonist, W13, inhibit insulin-stimulated glucose transport in adipocytes. These antagonists have two major effects on this process. Both BAPTA-AM and W13 cause a partial inhibition of insulin-stimulated Akt activation. In addition, both of these antagonists block the fusion of GLUT4 vesicles with the plasma membrane. These data suggest that calcium/calmodulin plays a central role both in the insulin signalling pathway and in the transport mechanism that allows GLUT4 vesicles to dock and fuse with the plasma membrane.

## WHAT ARE THE SITES OF INSULIN RESISTANCE? LESSONS FROM DESIGNER MICE

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Type 2 diabetes is currently viewed as a complex metabolic disorder with multiple causes. Its metabolic hallmarks are peripheral insulin resistance and impaired insulin secretion. The two defects are intimately intertwined in most diabetic patients. The nature of the fundamental defect remains unclear. While it is widely held that a post-receptor defect in insulin action could account for the protean manifestations of insulin resistance, the mechanism by which impaired insulin action affects beta cell function is not agreed upon. Insulin promotes a wide range of functions in multiple target cells by activating several kinases. They include the insulin receptor tyrosine kinase, as well as lipid kinases and serine/threonine kinases. The latter probably act as effectors of insulin action, resulting in modulation of enzyme activities, gene expression, cellular trafficking and proliferation.

Insulin resistance is a genetically complex trait, i.e., it is not inherited in a Mendelian fashion. Thus, simple genetic models, such as studies of candidate genes, have failed to identify the gene(s) responsible for insulin resistance. It is possible that, because of genetic heterogeneity, different genes are responsible for insulin resistance in different patients. Furthermore, the functional impairment caused by some genetic variations might be too subtle to be detected by the assays at our disposal, or may require the combined interactions of other mutant genes to give rise to the diabetic phenotype. In recent years, mice bearing targeted gene mutations that affect insulin action and beta cell function have contributed important new information to our understanding of the pathogenesis of insulin resistance and type 2 diabetes.

In this lecture, I shall review phenotypes of gene-targeted mice that have provided new insight into the mechanisms of insulin resistance and type 2 diabetes. First, I will address the question of whether insulin resistance represents a generalized impairment of insulin action or is restricted to specific organs. To this end, I will use as a paradigm mice with tissue-specific mutations of insulin and igf-1 receptors to demonstrate how insulin sensitivity is determined by canonical and non-canonical insulin target tissues. Genetic ablation experiments in mice indicate that non-canonical insulin target tissues, primarily liver and beta cells, play a key role in insulin action. To support this view, I will describe a novel transgenic-knockout mouse model generated in my laboratory. These mice express insulin receptors exclusively in liver and pancreatic beta cells. The phenotype of these mice provides important clues to the onset of insulin resistance and its compensatory mechanisms. Using crosses among mice with different diabetes susceptibility alleles, I will then try to demonstrate that type 2 diabetes is an oligogenic disease, and that genetic heterogeneity gives rise to pathogenetically distinct subtypes of diabetes, each one of which is likely to require different treatment options.

## STRESS HYPERGLYCEMIA IN THE CRITICALLY ILL : ADAPTATION OR DYSFUNCTION ?

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Hyperglycemia and insulin resistance are common in fed critically ill patients. There is no consensus about the optimal level of metabolic control with insulin in such patients. In most ICUs, insulin is only administered when blood glucose levels become excessively elevated (> 12 mmol/L).

We recently performed a prospective, randomized, controlled study to address this question. All mechanically ventilated, adult patients admitted to our tertiary level ICU were included, after informed consent from the closest family member. Only 5 patients participating in another study and 9 who were moribund or DNR coded at ICU admission were excluded. At admission, patients were randomized to either strict normalization of blood glucose levels (4.5-6.1 mmol/L) with continuously infused insulin during ICU stay or the conventional regimen in ICUs, which comprises insulin when blood glucose exceeds 12 mmol/L in which case glycemia was clamped to 10-12 mmol/L. An interim safety analysis revealed a difference in mortality and the study was ended for ethical reasons.

A total of 1548 patients were included, 765 in the intensive insulin schedule (IIS), 783 in the restrictive insulin schedule (RIS). The 2 groups were comparable at inclusion for all factors influencing outcome including age, gender, BMI, type of illness, severity scores (APACHE-II and TISS), secondary referral, history of diabetes (13%) and malignancy. Analyses were made on intention to treat basis.

IIS reduced ICU mortality by 43% (P=0.005) [35 deaths in the IIS group versus 63 deaths in the RIS group; death odds ratio for IIS, corrected for all baseline univariate predictors of ICU death, was 0.52 (0.33-0.82), P=0.004] and hospital mortality by 34% (P=0.01). Mortality reduction occurred exclusively in long-stay ICU patients and was due to prevention of death from multiple organ failure with sepsis. IIS also reduced the incidence of blood stream infections by 46%, renal failure requiring dialysis or hemofiltration by 42%, red cell transfusion requirements by 50% and critical illness polyneuropathy by 44%, together reducing duration of mechanical ventilatory support and intensive care.

In conclusion, the data suggest that disturbances in glucose metabolism during critical illness are not "adaptive and beneficial" since strict metabolic control with exogenous insulin substantially reduces morbidity and mortality.

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## HORMONES HYPERTENSION AND HEART FAILURE: NEW LIFE FOR ALDOSTERONE

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Two clinical findings, on a background of provocative clinical and experimental studies, have (re)established aldosterone as a key contributor to the pathophysiology of hypertension and heart failure. First, pioneering studies by Gordon and Stowasser have established that primary aldosteronism accounts for up to 15% of erstwhile 'essential' hypertension, a finding now being replicated around the world. Secondly, in September 1999 the results of the RALES trial were published. Low (26 mg/day) dose spironolactone added to current best-practice therapy for moderately severe heart failure provided a 30% improvement in mortality, and 35% in hospitalization. In considering these findings, the 'new biology' of aldosterone will be reviewed. To raise blood pressure, aldosterone has to activate mineralocorticoid receptors (MR) in the brain, specifically the A3V3 region. Non-epithelial MR are also found in the heart, where activation by aldosterone on a high salt background leads to cardiac hypertrophy and fibrosis. Vascular smooth muscle cells have epithelial-type (i.e. 'protected' by the enzyme 11 $\beta$ HSD2) MR; rapid, nongenomic effects of aldosterone via such MR include activation of the Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE); the mineralocorticoid-induced coronary vasculitis which triggers perivascular and interstitial cardiac fibrosis is equivalently blocked by MR or NHE-1 blockade. Finally, there is both clinical and animal experimental evidence for the failing heart being able to synthesize aldosterone, adding the possibility of paracrine, truly non-endocrine, actions in congestive heart failure.

## FAMILIAL CORTICOSTEROID-BINDING GLOBULIN DEFICIENCY DUE TO A NOVEL NULL MUTATION: ASSOCIATION WITH FATIGUE AND RELATIVE HYPOTENSION

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Corticosteroid-binding globulin is a 383-amino acid glycoprotein that serves a hormone transport role and may have functions related to the stress response and inflammation. We describe a 39-member Italian-Australian family with a novel complete loss of function (null) mutation of the corticosteroid-binding globulin gene. A second, previously described, mutation (Lyon) segregated independently in the same kindred. The novel exon 2 mutation led to a premature termination codon corresponding to residue - 12 of the pro-corticosteroid-binding globulin molecule (c.121G→A). Among 32 family members there were 3 null homozygotes, 19 null heterozygotes, 2 compound heterozygotes, 3 Lyon heterozygotes, and 5 individuals without corticosteroid-binding globulin mutations. Plasma immunoreactive corticosteroid-binding globulin was undetectable in null homozygotes, and mean corticosteroid-binding globulin levels were reduced by approximately 50% at  $18.7 \pm 1.3 \mu\text{g/ml}$  (reference range, 30-52  $\mu\text{g/ml}$ ) in null heterozygotes. Morning total plasma cortisol levels were less than 1.8  $\mu\text{g/dl}$  in homozygotes and were positively correlated to the plasma corticosteroid-binding globulin level in heterozygotes. Homozygotes and heterozygote null mutation subjects had a high prevalence of hypotension and fatigue. Among 19 adults with the null mutation, the systolic blood pressure z-score was  $12.1 \pm 3.5$ ; 11 of 19 subjects (54%) had a systolic blood pressure below the third percentile. The mean diastolic blood pressure z-score was  $18.1 \pm 3.4$ ; 8 of 19 subjects (42%) had a diastolic blood pressure z-score below 10. Idiopathic chronic fatigue was present in 12 of 14 adult null heterozygote subjects (86%) and in 2 of 3 null homozygotes. Five cases met the Centres for Disease Control criteria for chronic fatigue syndrome. Fatigue questionnaires revealed scores of  $25.1 \pm 2.5$  in 18 adults with the mutation vs.  $4.2 \pm 1.5$  in 23 healthy controls ( $P < 0.0001$ ). Compound heterozygosity for both mutations resulted in plasma cortisol levels comparable to those in null homozygotes. Abnormal corticosteroid-binding globulin concentrations or binding affinity may lead to the misdiagnosis of isolated ACTH deficiency. The mechanism of the association between fatigue and relative hypotension is not established by these studies. As idiopathic fatigue disorders are associated with relatively low plasma cortisol, abnormalities of corticosteroid-binding globulin may be pathogenic.

## ENDOCRINOLOGY OF AGEING

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## OUTCOMES AFTER SURGERY FOR PITUITARY ADENOMAS

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Non-functioning pituitary adenomas most often present as macroadenomas and cause visual field deficits and hypopituitarism. Of patients presenting with visual deficits, surgery improves visual loss in approximately 87%. Postoperative worsening of vision occurs in 4% of patients and in the remainder vision is unchanged. Twenty seven percent of patients presenting with hypopituitarism experience postoperative normalization of hormone secretion. Operative mortality for these larger, and often, more invasive, tumors is higher than for the hyperfunctioning adenomas and reaches just over one percent. For similar reasons, tumor recurrence is also an issue. Ten-year recurrence/persistence rates are approximately 16%, although only 6% require reoperation. Long-term follow up finds 83% of patients alive and well without evidence of disease.

Criteria for reporting remission from acromegaly require normalization of age adjusted IGF-1 levels; random growth hormone less than 2.5 nanograms per milliliter (ng/ml); and nadir growth hormone during an OGTT of less than 1 ng/ml. Using these strict criteria, transsphenoidal surgery obtains remission in 88% of those with microadenomas and 55% of macroadenomas. Acromegalic symptoms are improved in 95%. Recurrence at ten years is less than 2 percent. Ninety seven percent of patients have preserved normal pituitary function. Seventy-two percent of patients with greater than ten year follow-up, including those with adjunctive therapy, are alive and well without evidence of active disease.

Patients with prolactinomas who present for surgery are most often those who have failed medical management. Prolactin levels are normalized in 87% of patients with microadenomas and 56% of those with macroadenomas. The recurrence rate among those patients who are normalized after a transsphenoidal operation is 13% at ten years. Preserved pituitary function occurs in all but 3%.

Surgical management of Cushing's disease achieves a 91% remission rate for patients with microadenomas, but falls to 65% for those with macroadenomas. Although up to twelve percent of adults may experience recurrence after ten years, a higher percentage of children develop recurrence of Cushing's disease. Adjunctive radiosurgery has achieved remission in approximately 68% of patients whose disease either did not remit following surgery or recurred.

During this first century of transsphenoidal surgery for pituitary adenomas, significant technical refinements have occurred and have broadened the scope of this versatile approach. As we enter the next millennium, advances in medical the radiotherapy will continue in parallel and will usher in new, more effective treatments for this complex disease.

## MEDICAL MANAGEMENT OF PITUITARY TUMOURS.

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Pituitary tumours are optimally managed by a multi-disciplinary team which includes physicians, surgeons, radiotherapists and histopathologists, with excellent chemical pathology, interventional radiology and genetics support. This symposium presentation aims to highlight recent advances and controversies in the field.

Diagnosis of pituitary Cushing's disease remains controversial. Inferior petrosal sinus sampling (IPSS) is a useful but invasive and expensive technique. Central:peripheral ACTH gradients reliably identify a pituitary or para-pituitary source, but lateralisation within the pituitary appears less certain. We present our experience with metyrapone pre-treated IPSS and repeated sampling, confirming the experience with CRH-stimulated IPSS regarding pituitary localisation. Midline lesions were suggested by alternating or lack of ACTH lateralisation. Hormonal lateralisation within the pituitary has assisted the surgeon in 25% of cases where no lesion was macroscopically evident, and in further cases of false localisation by MRI.

The management of acromegaly is undergoing re-assessment. Neurosurgical resection followed by radiotherapy and/or medical therapy if required is the current treatment of choice. The introduction of long-acting somatostatin analogues and a Growth Hormone receptor (GH-R) antagonist raise the possibility of primary medical management. There are no long-term or controlled data comparing medical and surgical outcomes. The relative benefits, side-effects and costs of each approach will be discussed.

Hypopituitarism is a common outcome of pituitary macroadenomata, with or without surgery or radiotherapy. The diagnosis of ACTH-deficiency has traditionally been based on the insulin tolerance test (ITT). Concerns about safety of the ITT and specificity of the Synacthen test have led us to develop an HPLC-based overnight metyrapone test; preliminary results will be presented. Individualisation of glucocorticoid replacement will be discussed. The recent approval of GH replacement for GH deficient adults raises many issues, an important one being the need for a self-regulated, National audit of the quality and costs of managing these complex, chronic illnesses. A case is made for an Australian and New Zealand pituitary tumour registry.

## MODERN ASPECTS OF RADIOTHERAPY IN THE MANAGEMENT OF PITUITARY TUMOURS

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Radiotherapy has been used in the treatment of pituitary tumours for over 50 years. This experience has demonstrated that radiotherapy can control the mass effect, and reduce the abnormal hormone levels, in the majority of patients over a long term basis. Whilst conventional fractionated radiotherapy has been the dominant treatment approach current methods aim to address some of the more negative features of that approach. For the hormonally active tumours the time course to benefit is prolonged, with for all tumours the inclusion of adjacent neural tissue in the intermediate to high dose component of treatment. This can include normal pituitary, hypothalamus and medial temporal lobes. Stereotactic radiosurgery demonstrates high tumour control, more rapid hormonal reduction, and the potential for less normal tissue coverage. For the larger macroadenomas stereotactic fractionated treatment with beam shaping is the current preferred method for treatment. Long term risks of neural damage are low with these approaches. New methods of surgical management and medical treatment will redefine however the population of patients requiring radiotherapy in the future.

# ORAL FREE COMMUNICATION ABSTRACTS

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## REGULATION OF P450 AROMATASE EXPRESSION BY TNF $\alpha$ IN HUMAN ADIPOSE STROMAL CELLS

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P450 aromatase catalyses the formation of estrogens from androgen precursors. Aromatase is expressed in breast adipose tissue where it has been implicated in the development and progression of breast cancer. One key regulator of aromatase expression in breast is tumour necrosis factor alpha (TNF $\alpha$ ), which is secreted by adipocytes, breast cancer cells and by macrophages infiltrating a tumour site. TNF $\alpha$  (in the presence of glucocorticoids) acts via the adipose-specific promoter I.4 to increase transcription of the *CYP19* gene (that encodes aromatase) and consequently increase the supply of estrogens to the tumour. Inhibition of this action of TNF $\alpha$  would therefore be beneficial in the treatment of estrogen-dependent breast cancer. The intracellular mechanisms by which TNF $\alpha$  induces aromatase expression, however, are unknown. This study aims to characterise these pathways. Human adipose stromal cells were obtained by collagenase digestion of subcutaneous adipose tissue obtained from reduction mammoplasty procedures. Cells were cultured until confluent then treated with TNF $\alpha$  (5 ng / ml) in the presence of dexamethasone (DEX, 250nM) alone or in the presence of a MEKK1 inhibitor (U0126), p38MAPK inhibitor (SB203580) or a NF-kB inhibitor (BAY 11-7082) for 24 Hrs. Aromatase activity was subsequently measured as the rate of formation of  $^3\text{H}_2\text{O}$  from [ $^3\text{H}$ ] androstenedione. TNF $\alpha$ /DEX treatment induced aromatase activity 15-20 fold. In the presence of the MEKK1 inhibitor (U0126) aromatase activity was inhibited by only 20-30%. However both the p38 MAPK inhibitor (SB203580) and the NF-kB inhibitor (BAY11-7082) dose-dependently inhibited aromatase activity, reaching 80-90% inhibition at 20 $\mu\text{M}$  inhibitor. To assess the effects of these compounds on expression of *CYP19* mRNA, a quantitative real-time PCR assay was developed. TNF $\alpha$ /DEX treatment increased *CYP19* mRNA levels from 10 to 340 fg/ $\mu\text{g}$  total RNA. Again, this induction was dose-dependently and completely inhibited by both SB203580 and BAY11-7082, but only modestly inhibited by U0126. A similar profile of inhibition was also observed using 3T3-L1 preadipocytes transiently transfected with a luciferase reporter gene driven by 800 bp of *CYP19* promoter I.4, suggesting that SB203580 and BAY11-7082 inhibit aromatase activity at the level of *CYP19* gene transcription. Inhibition of *CYP19* transcription by BAY11-7082 suggests a role for the transcription factor NFkB in transducing the effects of TNF $\alpha$ . Consistent with this, treatment of adipose stromal cells with TNF $\alpha$  (5ng / ml) produced a 4-5 fold increase in NFkB mRNA levels. In addition, activity of an NFkB – responsive reporter gene transfected into 3T3-L1 preadipocytes was increased 5-6 fold in the presence of TNF $\alpha$ . These data suggest a role for both p38 MAP kinase and NFkB in TNF $\alpha$ -induced aromatase expression. These pathways may therefore represent novel targets for the development of more specific aromatase inhibitors for use in breast cancer therapy.

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## A POTENTIAL MECHANISM FOR GROWTH RETARDATION IN PREGNANCIES COMPLICATED BY ASTHMA

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In Australia, 12% of pregnancies are complicated by asthma, which increases the risk of low birth weight. The mechanisms causing this outcome are unknown. To investigate whether changes in placental function may contribute to this outcome, we measured fetal cortisol concentration in the umbilical vein at delivery, 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ -HSD2) enzyme activity (radiometric conversion assay), protein levels (western blot) and mRNA abundance (quantitative real-time RT-PCR) in placenta and analysed results in relation to inhaled glucocorticoid intake. Placental 11 $\beta$ -HSD2 metabolises cortisol to inactive cortisone and protects the fetus from high levels of maternal glucocorticoids. Decreased activity of this enzyme has been associated with intrauterine growth restriction. We questioned whether changes in 11 $\beta$ -HSD2 activity and fetal growth in asthmatic pregnancies may be associated with alterations in the insulin-like growth factor (IGF) axis. In particular, we investigated placental expression of IGF-I and IGF-II, which induce cell growth, and IGF binding protein 1 (IGFBP-1), which modulates IGF-I and IGF-II binding to their receptors. Two groups of pregnant asthmatic women were recruited into the study. One group (n=16) did not use any inhaled glucocorticoids for treatment (nil), while the other group (n=45) used inhaled glucocorticoids to control their asthma (GC). Outcomes in these women were compared to a non-asthmatic control group (n=11). Untreated asthma resulted in a 34% reduction in birth weight centile and a significant elevation in fetal cortisol levels compared to the control group. In addition, there was a significant reduction in 11 $\beta$ -HSD2 activity compared to controls (Student's t-test,  $P<0.05$ ). There were no differences in 11 $\beta$ -HSD2 mRNA abundance, but protein levels were significantly elevated in asthmatic placenta (Student's t-test,  $P<0.05$ ). Paradoxically, the use of inhaled glucocorticoids for asthma treatment restored birth weight centile and 11 $\beta$ -HSD2 enzyme activity to control levels. Fetal cortisol concentration was also restored to control levels, in a dose-dependent manner. By contrast, 11 $\beta$ -HSD2 mRNA and protein were not altered by glucocorticoid intake. Consistent with these data, there was an inverse correlation between fetal cortisol concentration at delivery and placental 11 $\beta$ -HSD2 activity across all groups. IGF-I mRNA, IGF-II mRNA and IGFBP-1 mRNA were reduced in all asthmatic placenta compared to controls, with IGFBP-1 mRNA being less affected than IGF-I mRNA. These changes were not reversed by glucocorticoid treatment, suggesting that alterations in the IGF axis are not directly responsible for the reduction in fetal growth observed in the untreated asthmatics. However, placental 11 $\beta$ -HSD2 activity was inversely correlated with IGFBP-1 mRNA, suggesting that alterations in 11 $\beta$ -HSD2 activity itself may be associated with some changes in growth factor pathways. This is the first study to show an association between reduced placental 11 $\beta$ -HSD2 activity and decreased birth weight in asthmatic pregnancies. This may be the result of inflammation, since a reversal of changes in placental function was observed in women using inhaled glucocorticoids for asthma treatment. These data suggest that alterations in the IGF axis are not responsible for observed changes in fetal growth, but that the activity of 11 $\beta$ -HSD2 is a crucial factor involved in fetal development in asthmatic pregnancies.



## MATRIX METALLOPROTEINASE EXPRESSION IS CORRELATED WITH INCREASED HISTOLOGICAL GRADE, MYOMETRIAL AND VASCULAR/LYMPHATIC INVASION IN ENDOMETRIAL CARCINOMA.

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Endometrial cancer is the most common female genital tract cancer and its metastasis is a key factor related to poor prognosis. Matrix metalloproteinases (MMPs) are a family of proteases that degrade components of the extracellular matrix. Most MMPs are secreted as pro-enzymes and activated extracellularly. MMPs have been implicated in tumour progression. In particular, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are commonly upregulated in cancers. Their presence in tumours is linked with invasion and metastasis due to their ability to degrade type IV collagen, the major constituent of the basement membrane. The expression of membrane-type 1 MMP (MT1-MMP) on the cell surface can induce specific activation of latent MMP-2. MMPs are specifically inhibited by the tissue inhibitors of metalloproteinases (TIMPs). However, TIMPs have additional functions in cell cycle proliferation and cell survival and TIMPs-1 and -2 have roles in controlling activation of proMMP-9 and proMMP-2 respectively. The present study was designed to a) define the cellular localisation of MMP-9, MMP-2 and MT1-MMP protein and mRNA in endometrial cancers using immunohistochemistry and *in situ* hybridisation respectively and to correlate staining scores with clinicopathologic features such as histological grade (1-3) and myometrial/vascular invasion b) define the cellular localisation of TIMPs 1-3 in the same tissues using immunohistochemistry and c) determine if the gelatinases are in their active forms using *in situ* zymography. MMP-9 and MMP-2 were detected in all 29 tissues examined while MT1-MMP was detected in 20/29 tissues. MMP-2, MMP-9 and MT1-MMP proteins were predominantly localised to tumor epithelial cells in comparison to stromal cells (MMP-9  $p < 0.0001$ , MMP-2 and MT1-MMP  $p < 0.05$ ). MMP-9 and MMP-2 mRNA were also predominantly localised to tumor epithelial cells. Furthermore, in the case of the gelatinases, increased staining scores were associated with higher tumour histological grades (MMP-9  $p < 0.0001$ , MMP-2  $p < 0.05$ ). MMP-9 and MT1-MMP were also associated with the presence of myometrial and vascular/lymphatic invasion (MMP-9  $p < 0.0001$ , MT1-MMP  $p < 0.05$ ). To determine whether the enzymes were present in their active forms, *in situ* zymography was performed using a gelatin substrate which fluoresces when cleaved at MMP-specific sites. Substantial gelatinase activity was demonstrated even at the early stages of tumour progression (ie. histological grades 1 and 2). TIMP-1 was immunolocalised to the stromal compartment in all grades of endometrial carcinoma, with variable tumour epithelial cell localisation being observed in grade 2 and 3 carcinomas only. Tumour cells from all histological grades stained intensely for TIMP-2 and -3 with varying degrees of stromal staining also being observed. Overall these results demonstrate elevated levels of the gelatinases in endometrial carcinoma. Their mechanisms of regulation are currently being investigated. (LAD is supported by a Monash Graduate Scholarship, LAS & JZ are supported by NH&MRC)

## PLACENTAL RESTRICTION INCREASES POSTNATAL GROWTH RATE AND SENSITIVITY TO IGF-I IN THE NEONATAL LAMB

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Most children who are short or light at birth due to intrauterine growth restriction (IUGR) exhibit accelerated growth in infancy ("catch-up" growth), but the physiological basis for this is unknown. The major axes involved in postnatal growth and its regulation are the insulin, insulin-like growth factors (IGF-I, II) and growth hormone axes. Because circulating levels of these anabolic hormones are reduced or at best normal in infants undergoing catch-up growth after IUGR, increased sensitivity to rather than abundance of these hormones may be responsible. Consistent with this, we have shown previously that fetal growth restriction and reduced size at birth in sheep, results in catch-up growth, which is associated with increased sensitivity to insulin. We further hypothesised that restriction of placental growth, which is a major determinant of fetal growth in late gestation, would reduce size at birth and increase sensitivity to IGF-I postnatally. Placental growth was restricted in sheep by removal of endometrial caruncles prior to mating (placental restriction, PR). Birth phenotype and postnatal growth (0-45 days) (38 control, 28 PR), and circulating IGFs and metabolic sensitivity to IGF-I *in vivo*, assessed by hyper-IGF-I euglycaemic clamp (3ug/kg/min) (19 lambs: 17 control, 2 PR) at 40 days of age, were measured. Placental restriction reduced size at birth for: weight (-24%), placental weight (-26%), crown-rump length (CRL) (-9%), body mass index (-12%), femur and tibia lengths (-6%), hindlimb circumference (-12%), and abdominal circumference (-10%) ( $n=66$ ,  $p < 0.05$ ). IGF-I sensitivity of glucose metabolism increased with decreasing birthweight ( $r^2=0.25$ ,  $n=19$ ,  $p < 0.025$ ). Fetal growth restriction increased rates of postnatal growth (fractional) of long bones (shoulder height and femur length), and each of these correlated positively with sensitivity to IGF-I of glucose metabolism ( $p < 0.05$  for both). Placental restriction decreased plasma concentrations of IGF-I and IGF-II and plasma IGF-I concentrations correlated positively with weight ( $p < 0.01$ ) and shoulder height ( $p < 0.05$ ) at birth. Rates of fractional growth for long bones (femur and tibia length) correlated positively with plasma IGF-II concentrations ( $p < 0.01$  and  $p < 0.05$ , respectively). Fetal growth restriction in sheep causes catch-up growth in the neonate, characterised by increased IGF-I sensitivity of glucose metabolism and occurs despite reduced concentrations of basal plasma IGF-I and II. Therefore neonatal catch-up growth after IUGR is associated with increased sensitivity to both insulin and IGFs and appears to occur to the extent allowed by the prevailing abundance of these hormones.

## STAGE SPECIFIC EXPRESSION OF GENES ASSOCIATED WITH RAT SPERMATOGENESIS: CHARACTERISATION BY LASER-CAPTURE MICRODISSECTION AND REAL-TIME PCR

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Spermatogenesis in the rat can be divided into fourteen morphologically distinct stages (stages I – XIV). Stage-specific expression of proteins during spermatogenesis has been demonstrated by immunolocalisation in whole sections and/or dissection of discrete segments of isolated seminiferous tubules. Due to the complexity of the seminiferous epithelium, quantitative analyses of stage-specific and cell-specific gene expression has hitherto proven difficult. The recent development of laser-capture microdissection (LCM) now allows for the isolation of individual cell populations from defined stages, which can then be processed for the quantitative analysis of gene expression by RT-PCR. The aim of this study was to quantitate the expression of spermatogenesis-related genes in frozen sections of whole seminiferous tubules at defined stages of spermatogenesis isolated using LCM from testis sections of normal adult rats. The genes studied were transition protein – 1 (TP-1, a protein expressed by elongating spermatids), androgen receptor (AR, expressed by Sertoli, peritubular and Leydig cells), and  $\beta$ 1-integrin (found at Sertoli cell – germ cell and Sertoli cell – Sertoli cell junctions). Frozen sections (10 $\mu$ m) were obtained from normal adult rat testes, fixed in acetone, and immediately processed for LCM. To obtain sufficient material for analysis, 15 tubule cross-sections were captured by LCM from defined stages of spermatogenesis (I-V, VII-VIII, and IX-XIII), with n = 3 replicates per stage grouping. Total RNA was extracted using TRIzol reagent and RNA mass was quantitated by a fluorescence-based microplate RNA assay against a standard RNA preparation of defined mass (inter-assay cv = 11.6%, n = 14 assays). RNA (8ng per sample) was then reverse transcribed using AMV RT, with the absence of genomic DNA confirmed by RNA samples processed lacking AMV RT enzyme. The ability to reverse transcribe small quantities of RNA ( $\geq$  0.39ng) using AMV RT was previously validated. Gene expression for TP-1, AR, and  $\beta$ 1-integrin was then quantitated by real-time PCR using the Roche Lightcycler with a rat testicular cDNA preparation of arbitrary unitage as the standard. PCR conditions for each gene were optimised to ensure amplification of a single product species, which was confirmed by agarose gel electrophoresis and sequencing of selected samples. TP-1 expression was determined to be lowest during stages I-V, increased 3.6-fold at stages VII-VIII and remained high during stages IX-XIII (p < 0.01). AR expression was highest at stages VII-VIII compared with stages I-V and IX-XIII, while  $\beta$ 1-integrin expression was highest at stages IX-XIII, however these differences did not achieve significance. For TP-1 and AR, these results confirm previous reports of stage specific expression, whereas  $\beta$ 1-integrin expression has not been previously characterised. We conclude that the stage-specific expression of testicular genes can be quantitated by LCM and real-time PCR. Future studies will extend these analyses to examine the expression of genes in specific cell populations from defined stages from normal rats and from animals in which the hormonal regulation of spermatogenesis has been manipulated.

## THE N-TERMINAL DOMAIN OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-6 (IGFBP-6) CONFERS ITS INSULIN-LIKE GROWTH FACTOR-II (IGF-II) BINDING SPECIFICITY

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The insulin-like growth factors, IGF-I and IGF-II, are mitogenic peptides involved in growth and development. IGF actions are modulated by a family of six structurally related high-affinity IGF binding proteins (IGFBP 1-6). The cysteine-rich N- and C-terminal domains of the IGFBPs share considerable sequence homology between binding proteins, and previous studies of IGFBPs have shown IGF binding to both of these domains. IGFBP-6, the subject of this study, differs from the other IGFBPs in that it binds IGF-II with the highest affinity of any IGFBP and IGF-I with ~60-fold lower affinity. The aim of this study was to determine the contribution of the N- and C-terminal domains to the IGF-II specificity of IGFBP-6.

The full-length protein (IGFBP-6<sup>28-240</sup>), N-terminal (IGFBP-6<sup>28-110</sup>) and C-terminal domains (IGFBP-6<sup>161-240</sup>) were cloned and expressed in *E.coli* using the pProEx His<sub>6</sub>-tag system. Bacteria were lysed by sonication under denaturing conditions (3M guanidine HCl) and purified by Ni-NTA affinity chromatography. Identities of the constructs were confirmed by mass spectrometry. Real-time binding kinetics was investigated by surface plasmon resonance using BIAcore. The IGFBP-6 constructs were immobilised using the amine-coupling method. IGF-I and -II (1-100nM) were passed over the chip at a flow rate of 10  $\mu$ l/min. The binding data were fitted to the Langmuir 1:1 model using the BIAevaluation software to calculate kinetic constants.

The BIAcore results confirm the expected IGF-II binding preference for full length IGFBP-6, with an 18-fold greater affinity evident for IGF-II ( $K_D = 7.5 \times 10^{-10}$  M) than IGF-I ( $K_D = 1.4 \times 10^{-8}$  M). Interestingly, the majority of IGF-II binding was seen in the N-terminal domain ( $K_D = 2.7 \times 10^{-8}$  M) which displayed a 300-fold greater affinity for IGF-II than IGF-I. The IGF-II association rate for the N-domain ( $k_a = 3.6 \times 10^5$  M<sup>-1</sup>s<sup>-1</sup>) was comparable to that seen for full length IGFBP-6 ( $k_a = 9.5 \times 10^5$  M<sup>-1</sup>s<sup>-1</sup>), whereas the dissociation rate for the N-domain ( $k_d = 9.6 \times 10^{-2}$  s<sup>-1</sup>) was markedly faster compared to full-length IGFBP-6 ( $k_d = 7.1 \times 10^{-4}$  s<sup>-1</sup>), resulting in a 35-fold reduced overall affinity. N-domain binding to IGF-I and C-domain binding to both IGF-I and -II were all >1000-fold weaker compared to full-length IGFBP-6. In conclusion, the N-terminal domain of IGFBP-6 has a marked IGF-II binding preference. However, its reduced IGF affinity compared to full-length IGFBP-6 implies that cooperative binding between different sites in IGFBP-6 is necessary to produce high-affinity IGF binding.

## AMIODARONE-INDUCED THYROTOXICOSIS: EVALUATION OF COLOUR FLOW DOPPLER SONOGRAPHY IN PREDICTING THERAPEUTIC RESPONSE

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Amiodarone-induced thyrotoxicosis (AIT) presents a therapeutic challenge because of its resistance to standard antithyroid medications. In iodine-deficient regions, colour flow Doppler sonography (CFDS), among other parameters, has been reported to distinguish between two types of AIT. Type I AIT, associated with increased vascularity (CFDS patterns I - III), responded to antithyroid drugs while type II AIT, associated with no/little thyroid vascularity (CFDS pattern 0) responded to prednisolone. We sought to clarify if CFDS was useful in predicting treatment outcomes in a retrospective study (1998 - 2000) of 25 patients with AIT in an iodine-replete environment. Fourteen of 25 patients showed CFDS 0. Twelve of these 14 were evaluable for prednisolone responsiveness, of whom 7 (58%) were prednisolone-responsive. Of 11 patients with CFDS I - III, 4 (36%) responded to antithyroid medication alone and only 1 of 6 (17%) was prednisolone-responsive. Because of medical treatment failure, 7 of the 25 (28%) patients from both CFDS groups required urgent near-total thyroidectomy which was well tolerated. These findings suggest that CFDS is useful in the management of AIT in that CFDS 0 correlates better with prednisolone response (58%) than does CFDS I - III (17%). However, unlike experience in iodine-deficient regions, CFDS alone does not permit categorisation of AIT into two specific types that correlate absolutely with response to thionamide or prednisolone. Therefore, although CFDS 0 does not consistently predict prednisolone responsiveness, the presence of flow appeared predictive of non-response to prednisolone. In addition, early recourse to near-total thyroidectomy may be warranted, as previously reported (1), and allows continuation or resumption of amiodarone as indicated for cardiac reasons.

## PREVALENCE OF THYROID ANTIBODIES IN A COMMUNITY POPULATION

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The Busselton community in the southwest of WA has been investigated by cross-sectional health surveys since 1966. The current retrospective study of participants in the 1981 cross-sectional survey of adults in Busselton was designed to determine the prevalence of thyroid antibodies (anti-thyroid peroxidase, ATPO and anti-thyroglobulin, ATG) in a general population. With the advent of newer more sensitive and specific assays for ATPO and ATG, we hypothesized that more subjects with autoimmune thyroid disease would be detected than in the Wickham Survey<sup>1</sup> (where the prevalence of elevated thyroid antibodies anti-microsomal and anti-thyroglobulin agglutination assays was 11.2% females (F) and 2.8% males (M)). All assays were performed on an Immulite 2000 chemiluminescent immunoassay analyzer on samples from 1981 stored at -80C. The total study group contained 2117 subjects (1050 F and 1067 M) aged between 16.9 - 88.9 years. Elevations of ATPO and/or ATG occurred in 15.1% of the study group. An elevated ATPO occurred with a prevalence of 11.4% (17.3% F, 6.8% M) and for ATG it was 8.5% (11.4% F, 5.5% M). We analysed the group in three subsets (i-iii) as follows:

Prevalence (%) of thyroid antibodies in:	ATPO > 35IU/L		ATG > 40IU/L		Both elevated	
	F	M	F	M	F	M
(i) 74 subjects (61 F, 13 M) with a stated history of thyroid disease	27	8.3	3.2	8.3	21	17
(ii) 173 subjects (109 F, 64 M) without history of thyroid disease but abnormal FT4 and/or TSH	24	4.3	2.7	5.7	24	27
(iii) 1870 subjects (880 F, 990 M) without history of thyroid disease and biochemically normal FT4 and TSH	6.5	3.3	4.0	2.0	4.4	1.6

While there was no significant change with age in TSH levels in males or females, FT4 concentrations declined significantly ( $p < 0.01$ ) in both sexes and anti-TG concentrations rose significantly ( $p < 0.05$ ) in males. We conclude that (1) the prevalence of thyroid antibodies (ATPO and ATG) in both females and males in Busselton in 1981 was significantly higher than observed in the earlier Wickham Survey; (2) the ratio of females to males with elevated thyroid antibodies was 2-3, much lower than previous studies have suggested, and (3) these data provide renewed interest in testing for ATG, as elevated ATG antibodies alone identified more than 25% subjects in the thyroid antibody positive group.

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## POSTPARTUM MATERNAL IODINE STATUS AND THE RELATIONSHIP TO NEONATAL THYROID FUNCTION.

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**Aim:** Iodine deficiency in the postpartum has the potential to adversely affect neonatal neuropsychointellectual development. We assessed maternal iodine status to determine its relationship to neonatal thyroid function.

**Methods:** We measured urine and breast milk iodine in 50 postpartum women and TSH in their infants. Iodine was measured by inductively coupled plasma mass spectrometry. TSH was measured by fluoro immunoassay on a heel-prick blood sample as a part of the routine neonatal screening programme.

**Results:** Moderate to severe iodine deficiency as defined by urine iodine < 50 ug/L was found in 29 of the 50 subjects (58%). The mean  $\pm$  1 SD urine iodine was  $46.8 \pm 28.5$  ug/L or  $86.6 \pm 45.6$  ug/g creatinine. The median (range) breast milk iodine was 84.0 (25 – 234) ug/L. Breast milk iodine was significantly correlated with urine iodine expressed as ug/g creatinine ( $r = 0.52$ ,  $p < 0.001$ ) but not with urine iodine expressed as ug/L ( $r = 0.19$ ,  $p = 0.20$ ). Six percent of neonates had TSH values of greater than 5 mIU/L. Neonatal TSH levels were positively correlated with breast milk iodine ( $r = 0.43$ ,  $p = 0.002$ ). There was no significant correlation between neonatal TSH levels and the mother's urine iodine content.

**Conclusion:** There is a high prevalence of iodine deficiency in these lactating postpartum women. Urine iodine expressed as ug/g creatinine is a good predictor of breast milk iodine content. In our study, higher breast milk iodine was correlated with a higher neonatal TSH. The impact of breast milk iodine on neonatal thyroid function and neuropsychointellectual development needs further study.

## INHALED GLUCOCORTICOID USE FOR THE TREATMENT OF ASTHMA DURING PREGNANCY DOES NOT AFFECT MATERNAL, PLACENTAL OR FETAL SYSTEMIC FUNCTION

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Severe asthma during pregnancy is associated with an increased risk of pre-term delivery and low birthweight. The mechanisms that cause these outcomes have yet to be defined. Inhaled steroids are recommended for the control of asthma during pregnancy, however glucocorticoids may adversely affect fetal growth. To determine whether inhaled steroids may have systemic effects upon the fetus, we examined adrenal function of the mother and fetus. We recruited a control group (n=28) and three groups of asthmatic women at the end of the first trimester of pregnancy. Asthmatics were classified into mild (n=34), moderate (n=21) or severe (n=29) groups based on lung function and asthma symptoms. Glucocorticoid intake during pregnancy was recorded and a cumulative dose for pregnancy assigned to each subject. Three blood samples were collected from each woman during pregnancy and plasma cortisol, osteocalcin, corticotropin releasing hormone (CRH) and estriol measured using radioimmunoassay. Baseline characteristics for control and asthmatic women and their neonates were not significantly different. However, maternal weight at the beginning of pregnancy was significantly increased in women using high doses of inhaled steroid. Forced expiratory volume at one second (FEV<sub>1</sub>) adjusted for height and weight was significantly reduced in women with severe asthma. Maternal plasma cortisol, CRH and estriol concentrations increased with gestation in all groups. These hormone levels did not vary with asthma severity or glucocorticoid intake. As an independent measure of the effects of inhaled steroid on the maternal system using a marker that does not vary diurnally or with gestation, osteocalcin concentrations were examined. Osteocalcin concentrations did not change as gestation progressed and concentrations in asthmatic groups regardless of severity of glucocorticoid intake were significantly increased when compared to controls subjects. These findings suggest that inhaled glucocorticoids do not affect maternal, placental or fetal systemic function during pregnancy. This is an important finding that confirms the safety of inhaled glucocorticoids in the treatment of asthma during pregnancy.

## PARATHYROID VENOUS SAMPLING IN RECURRENT PRIMARY HYPERPARATHYROIDISM.

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The long term surgical cure rate for primary hyperparathyroidism is greater than ninety five percent. For those patients who have recurrent disease pre-operative localisation improves reoperation success rates. Selective parathyroid venous sampling (SPVS) for intact parathyroid hormone is particularly useful when non-invasive localisation techniques are negative. We retrospectively reviewed all known cases in our institution between 1994 and 2001 who had localisation procedures prior to reoperation for recurrent primary hyperparathyroidism. Ten patients underwent computer tomography (CT), nuclear imaging (MIBI), and SPVS. Results of these tests were compared with operative findings, histology and calcium levels post-operatively. Two out of 10 cases had localisation of a lesion on CT and MIBI imaging, yet had SPVS for reasons which related to the site of non-invasive imaging (e.g., a young female requiring open thoracotomy to access a mediastinal lesion). Seven out of 10 were negative on both MIBI and CT scans yet localised with SPVS. One case failed to localize on all three tests. All four cases reoperated to date have had positive correlation of SPVS with operative findings and histopathology. Three have had long term cure, and the other case has had multiple apparently successful parathyroid resections where the diagnosis is parathyroid seeding.

We conclude that Selective Parathyroid Venous Sampling has proved to be a safe and useful tool in localizing in recurrent primary hyperparathyroidism at our institution.

## VITAMIN D INSUFFICIENCY IN THE WESTERN AUSTRALIAN POPULATION

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The aims of the study were to measure the prevalence of vitamin D insufficiency in an unselected population of West Australians living in the community, to confirm the lack of an age-related decline in serum vitamin D concentration (25D), and to investigate the relationship between vitamin D insufficiency and secondary hyperparathyroidism.

400 subjects, (200 male) aged 30-70, were randomly selected, after exclusion of those with renal impairment, from 1500 volunteers who were recruited from the electoral roll for the cardiovascular Risk Factor Survey. Serum 25D concentrations were measured by radioimmunoassay (Diasorin), on samples stored at -70c (reference range 30 –120nmol/L). Vitamin D insufficiency was defined as 25D <50 nmol/L. Serum PTH was measured by chemiluminometric immunoassay (Immulite 2000, DPC).

Mean 25D was 72 (range18-150). 6 subjects had 25D < 30nmol/L and a further 70 had 25D between 30 and 50nmol/L, giving a prevalence of vitamin D insufficiency of 19%. There was no age-related decline in 25D. There appeared to be an inverse relationship between 25D and PTH levels.

We conclude that vitamin D insufficiency is not uncommon in the healthy population and that there is no age-related decline in 25D between ages 30 and 70 in healthy community dwellers. The rare occurrence of secondary hyperparathyroidism in those with a 25D > 50nmol/L, supports the view that a desirable 25D is >50nmol/L.

## EXPRESSION AND PROMOTER UTILISATION OF AROMATASE IN BENIGN AND MALIGNANT PROSTATE.

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Androgens regulate the growth and development of the prostate and are essential for the pathogenesis of prostate carcinoma (PCa), but as malignancy progresses, tumour growth becomes androgen independent. Increasing evidence suggests that estrogens have a role in the development of PCa. In the male androgens are converted to estrogens by the aromatase enzyme. Aromatase is expressed in a number of tissue types, and is regulated by tissue specific promoters. In breast cancer, the promoter switch from a glucocorticoid regulated I.4 to the cAMP regulated I.3 or PII has been identified. The resultant positive feedback loop and over-expression of aromatase is believed to play a crucial role in the development and progression of malignancy. Expression of aromatase in the prostate is controversial.

The aims of this study were to examine the expression of aromatase in the prostate, determine the promoter(s) used, and to show whether expression and promoter utilisation is altered in PCa. Using RT-PCR we have demonstrated the expression of aromatase in non-malignant prostate tissue, in the androgen dependent LNCaP cell line as well as the androgen independent DU145 and PC3 cell lines. Pure populations of non-malignant prostate epithelia, normal prostate stroma and PCa were obtained by Laser Capture Microdissection (LCM) and used for RT-PCR. Expression of aromatase was detected in all cell types.

Promoter utilisation was examined in benign prostate tissue and the PCa cell lines. Using relative quantitative real time RT-PCR, we have been able to demonstrate that promoters PII, I.4 and I.3 are utilised in benign prostate tissues; the dominant transcript (>50%) was PII. Promoter I.4 was dominant in the androgen responsive LNCaP cells, and was associated with a concomitant decrease in PII expression. The androgen independent DU145 and PC3 cells showed elevated promoter I.4 expression while PII was undetectable. These data demonstrate a switch from PII to promoter I.4 in the transition from benign to hormone independent tumour cells as malignancy progresses. Regulation of promoter I.4 occurs through the Jak/STAT pathway, which is responsible for growth factor and cytokine signalling.

These data support the hypothesis that estrogen, acting via cytokines through the Jak/STAT pathway, is involved in the development and/or the progression of prostate malignancy.

## HORMONAL REGULATION OF TISSUE KALLIKREIN 4 (KLK4) IN ENDOMETRIAL, BREAST AND PROSTATE CANCER CELL LINES.

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The tissue kallikreins (*KLKs1-15*) are a multi-gene family involved in a number of processes such as vascular permeability, blood pressure regulation, extracellular matrix (ECM) degradation and cell proliferation. A feature of this family is that many of these genes are expressed in hormone-dependent tissues, such as the prostate, breast and endometrium and additionally, have been shown to be hormonally regulated in cancer cell lines derived from these tissues<sup>1</sup>. *KLK2* and *KLK3* (or prostate-specific antigen/PSA) have been the most extensively studied, particularly with respect to androgen regulation at the transcriptional level, in prostate cancer cells. Several consensus and non-consensus androgen-response elements (AREs) have been identified. In addition, AREs, located within the proximal promoter and the far upstream enhancer regions act in synergism to regulate gene expression. Furthermore, androgen-independent mechanisms of *KLK2* and *KLK3* transcription have been identified thus increasing the complexity of this system. *KLK4*, a recently identified member of the family, has also been shown to be upregulated at the mRNA level by androgens in the LNCaP prostate cancer cell line<sup>2</sup> and by progesterone in the endometrial (KLE) and breast (BT474) cancer cell lines<sup>3,4</sup>. However, little is known of the mechanisms underlying this regulation. In addition, the *KLK4* promoter is yet to be characterised. Thus, the aim of this study was to: 1. Further examine the androgen and progesterone regulation of *KLK4* in LNCaP, KLE and breast cancer cells T47D, and 2. To characterise the first 2 kb of the *KLK4* promoter. Cell lines were treated with 10 nmol/L dihydrotestosterone (DHT) and progesterone over a 48 hr period. RT-PCR/Southern blot and Western blot analysis showed an increase in *KLK4* gene levels in LNCaP and T47D cells, while K4 protein levels were elevated in the LNCaP and KLE cell lines compared to the untreated control. Time-course studies and treatment with protein and RNA synthesis inhibitors are currently being performed to determine the transcriptional response of this gene. To determine the start of the promoter region, the transcriptional start site (TSS) is required. A number of variations on 5'RACE were performed, however these were not successful. Utilising FAM-labelled *KLK4* gene-specific primers, the identification of the TSS has been identified to be approximately 165 bp upstream from the start site of translation and is in agreement with recently published results<sup>5</sup>. A 2 kb region covering the TSS has been cloned into a Luciferase reporter gene vector and studies are underway to look at promoter activity. Transcription factor analysis of this 2 kb promoter region identified several consensus sequences such as the Sp1, c-Myb, c-Ets-1, and cAMP response elements, however, surprisingly no androgen or progesterone response elements were identified. Further studies using the reporter gene assay are being generated to identify *cis*-acting elements involved in the regulation of this gene.

## CELLULAR LOCALISATION OF *KLK4* IN THE PROSTATE AND ITS ASSOCIATION WITH PROSTATE CANCER PROGRESSION

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Prostate cancer is the most commonly diagnosed cancer and second leading cause of cancer deaths in older men. Although prostate specific antigen (PSA) is currently the most valuable tool available for the diagnosis of prostate cancer, it still lacks specificity. To overcome this problem, new markers with better specificity and sensitivity are needed. Recently, we and others identified a PSA-related enzyme, *KLK4*, which is also highly expressed in the prostate. Thus, **the aim of this study** was to determine the cellular localisation of the *KLK4* gene and protein in the prostate and its association with cancer progression.

Prostatic tissue was obtained as formalin-fixed and paraffin-embedded blocks from the archives of the Royal Brisbane Hospital, Queensland. Samples included 1 normal prostate, 11 benign prostatic hyperplasia (BPH), 20 prostate cancers and 3 bone metastases. Using *in situ* hybridisation with a digoxigenin (DIG)-labelled cRNA probe for *KLK4*, the presence of *KLK4* mRNA was detected in the secretory cells of the prostate gland in benign, prostatic intraepithelial neoplasia (PIN) tissues and in adenocarcinoma cells. This localisation was similar to that of *KLK2* and *KLK3*, which are restricted to the secretory cells of the glands. However, *KLK4* mRNA was also noted in basal cells of PIN lesions. This is in strong contrast to the pattern observed for *KLK2* and *KLK3* in which there is no expression in basal cells. *KLK4* expression was also seen in bone metastasis tissue from prostate cancer patients. The mRNA localisation of these three kallikreins was consistent with the protein expression detected by immunohistochemistry with K4 anti-peptide, PSA and hK2 antibodies. The hK2, PSA and K4 proteins were predominantly localised in the cytoplasm of the secretory cells of the benign, PIN and cancer glands. However, the K4 protein was also frequently expressed in the nucleus of the secretory and basal cells. This is an unusual finding, since these serine proteases are usually secreted and only localised to the cytoplasm. The cytoplasm of basal cells of PIN tissue also showed strong positive staining for K4. The level of expression of K4 staining appeared lowest in normal or benign prostatic hyperplasia glands, and increased with cancer progression, appearing highest in later stage adenocarcinoma. Similarly, K2 also showed an increased level of expression in cancer, in comparison to benign glands. This expression level is in contrast to PSA, cancer tissue showing lower PSA levels than normal and benign glands. The high expression of *KLK4* mRNA and K4 protein in PIN lesions and cancer is of interest, since PIN lesions are suggested to be pre-cancerous lesions. Further studies are underway to further examine the novel nuclear localisation and to more clearly define the association of K4 over-expression with prostate cancer progression and also to determine the usefulness of K4 as a diagnostic/prognostic marker for prostate cancer.

## EXPRESSION OF ADAMS PROTEASES IN PROSTATE CANCER CELL LINES AND THEIR REGULATION BY EPIDERMAL GROWTH FACTOR (EGF).

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The ADAMs are a multi-gene family of metalloproteases, some of which have been shown to play a role in diverse biological processes such as fertilization, myogenesis, neurogenesis and growth factor activation (eg. TNF-alpha). So-named because they possess both A Disintegrin And Metalloprotease domain, the ADAMs have potential implications for the metastasis of human tumour cells via cell adhesion and protease activities. We have recently reported (McCulloch et al (2000) Mol Cell Endocrinol 167:11) the expression of several ADAMs (at the RT-PCR level) in prostate cancer cell lines and their regulation by androgen. We now report the expression of ADAM -9 and -10 proteins in the metastatic LNCaP cell line variants C4, C4-2 and C4-2B as well as LNCaP, DU-145, ALVA-41 and PC-3 cell lines. Additionally, we examined the regulation of ADAM-10 protein by EGF in the prostate cancer cell line LNCaP. Western immunoblots revealed bands of the expected size for the active forms of ADAM-9 (79KDa) and -10 (100KDa) in all cell lines studied. Additionally in some cell lines bands potentially representing the pro-form of ADAM-10 were observed. A lower Mr band of 54KDa was also observed for ADAM 10 in all cell lines, this may represent a splice variant or a soluble form, consistent with the multiple mRNA transcripts also observed on Northern blots. Given the known regulation of matrix metalloproteases by EGF, LNCaP cells were incubated with increasing doses of EGF (0-50ng/ml) in serum-free medium for 48h. As expected this led to a significant up-regulation in LNCaP proliferation. ADAM -10 protein expression (100Kda) was measured by western immunoblot and was found to be also significantly up-regulated by EGF (50ng/mL) (using subsequent image quantitative analysis). This is the first description of the ADAMs protein expression in the above-mentioned prostate cancer cell lines and of ADAM-10 regulation by EGF. Given the potential functional capacity of ADAMs to mediate both extracellular matrix degradation via their metalloprotease domain and cell migration via their disintegrin domain, these observations further our understanding of the possible roles of ADAMs in the complex interactions underlying prostate cancer biology and metastasis.

## RECOMBINANT HUMAN GROWTH HORMONE EXACERBATES CHOLESTASIS IN ENDOTOXINAEMIC RATS.

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Human and animal studies have shown that growth hormone (GH) can potentiate the biological effects of endotoxin (LPS). Since the hepatic bile salt transport system is important in endotoxin processing we examined whether recombinant human GH (rhGH) modulates the cholestatic potential of LPS.

Adult Sprague-Dawley rats were sham-operated or implanted with an osmotic ALZET<sup>®</sup> pump infusing rhGH (Pharmacia & Upjohn) for 4 days. On day 4 they were injected with either 500 µg/100g *E. coli* LPS or saline. Animals were fasted until culling after 6 and 12 h. Serum was taken and liver samples were used for isolation of RNA, which was quantified by Northern analysis.

In animals exposed to LPS, serum total bilirubin, total bile acids and γGT were selectively induced in the GH-treated animals, indicative of cholestasis. Liver enzymes AST and ALT were increased 2- to 3-fold by GH, indicating exacerbation of hepatocellular damage. Increased IL-6 levels at 12 h in GH/LPS treated animals suggested that GH increased LPS toxicity. Serum levels of IL-6, a known inducer of transcription of many hepatic genes and a putative marker of outcome in critical illness, were highly correlated with the total bilirubin levels.

Bile acids are mainly secreted through the Na<sup>+</sup>-dependent bile transport system: Na<sup>+</sup>-taurocholate cotransporter (ntcp) basolaterally and sister of P-glycoprotein (spgp) apically, while bilirubin follows the Na<sup>+</sup>-independent pathway: organic anion transporting polypeptides (oatp)1/2 basolaterally and multidrug resistance-associated protein 2 (mrp2) apically. "Escape" transporters like multidrug resistance (mdr) 1b protect the hepatocyte against overflow of various toxic substances.

To study the molecular basis for cholestasis in a rat model of endotoxaemia we cloned the nine major bile transporter cDNAs by reverse transcriptase PCR from rat liver total RNA. Northern analyses showed a slight drop in ntcp mRNA, a 20% increase in spgp, and a 5-fold increase in mdr1b expression. Oatp2 and mrp2 gene expression showed no differential effect between sham and GH-treated LPS rats.

To examine whether hepatic insulin sensitivity might contribute to the altered liver bile salt processing, serum levels of insulin-regulated, hepatocyte-derived, IGF-binding protein-1 (IGFBP-1) were measured. IGFBP-1 was elevated in all LPS treated groups at 6 and 12 h, indicating decreased hepatic insulin responsiveness, but there was no additive effect of GH.

In conclusion, in this model of endotoxaemia, GH strongly induces cholestasis, with a marked upregulation of the protective "escape" transporters. While the regulating mechanism remains undetermined, the results suggest that impairment of the endotoxin-processing bile salt transport system by GH might contribute to its toxicity in endotoxaemia.

## OESTROGEN INHIBITS GH SIGNALLING BY SUPPRESSING GH-INDUCED JAK2 PHOSPHORYLATION

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Oral oestrogen administration attenuates the metabolic effect of GH in GH-deficient women, suggesting that oestrogen inhibits GH-induced gene expression. To investigate the mechanism involved, we studied oestrogen effects on GH activation of the JAK/STAT signalling pathways in HEK293 and T47D (breast cancer) cells. HEK293 cells stably expressing GH receptors (293GHR), were transiently transfected with oestrogen receptor and luciferase reporter constructs containing the binding elements for STAT3 or STAT5. Twenty four hours after transfection, the cells were treated with GH (500ng/ml) and varying concentrations of 17β-oestradiol (E<sub>2</sub>, 1-1000nM) for 6h before luciferase activity was measured.

In 293GHR cells, GH increased luciferase activity of the STAT3- and STAT5-reporters by 4-5 fold. Addition of E<sub>2</sub> resulted in a dose-dependent reduction in the activities of both reporters, to a maximum of 64±4% and 67±2% of control respectively (P<0.001) at 100nM. The inhibitory effect of E<sub>2</sub> was obliterated by co-treatment with the anti-oestrogen ICI182780. No reduction in GH transcriptional activity was seen when E<sub>2</sub> was added 1-2h after GH treatment. Similarly, the inhibitory effects of E<sub>2</sub> on GH action were seen in T47D cells. The effect of E<sub>2</sub> on JAK2 phosphorylation induced by GH in 293GHR cells was next studied by Western blotting. E<sub>2</sub> treatment significantly reduced GH-stimulated JAK2 phosphorylation to 45±5% of control (P<0.0005). This effect was attenuated by pre-treatment with actinomycin D, suggesting requirement of *de novo* gene expression. The inhibitory effect of E<sub>2</sub> was not affected by vandanaate, revealing that the action was not mediated by tyrosine phosphatase. These data provide the first evidence that oestrogen specifically inhibits GH signalling via the JAK/STAT pathways by suppressing JAK2 phosphorylation. This represents an important regulatory interaction between the JAK/STAT and sex steroid signalling pathways. (Supported by the NHMRC of Australia and Eli Lilly Australia)



## OESTROGEN REGULATION OF GH SIGNALLING IN HUMAN HEPATIC TISSUE: EFFECTS OF OESTROGEN RECEPTOR- $\alpha$ AND - $\beta$

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Oral administration of oestrogen increases circulating GH but reduces IGF-I levels. The dissociation of GH and IGF-I levels suggests that oestrogen inhibits GH action, but the mechanism of inhibition is unclear. The action of oestrogen is mediated by two oestrogen receptor (ER) subtypes, ER $\alpha$  or ER $\beta$ , which exhibit overlapping and distinct ligand binding and functional properties. In the present study, we investigated the effects of E<sub>2</sub> and the role of ER subtype function on GH signalling in human hepatic tissue. Human hepatoma cells (HuH7) were transiently transfected with human GH receptor (GHR), a luciferase reporter containing Stat5 binding element, and human ER $\alpha$  or ER $\beta$ .

GH treatment of HuH7 cells induced a slight but significant increase in reporter activity (13.5 $\pm$ 0.5%;  $P$ <0.05). Co-transfection with GHRs greatly enhanced GH-induced luciferase response to 2.7 $\pm$ 0.3 fold ( $P$ <0.05). The GH effects were concentration- and time-dependent, with maximal response observed at 6h incubation with 500ng/ml GH. 17 $\beta$ -Estradiol (E<sub>2</sub>) suppressed GH-induced luciferase response in a dose-dependent manner with significant suppression at 1nM. ER $\alpha$  was more potent than ER $\beta$  in mediating the inhibitory action of E<sub>2</sub>, with maximal suppression of 33 $\pm$ 6% and 21 $\pm$ 9% of control occurring at 1000nM, respectively ( $P$ <0.05). Selective oestrogen receptor modulators (SERMs) are a group of compound which exhibit mixed agonistic and antagonistic oestrogen effect upon binding to ER. To examine the effect of such compounds on GH action, we studied the effect of 4-hydroxytamoxifen (4-HT) on GH signalling. 4-HT did not affect GH induced transcriptional activity. Studies with a luciferase reporter containing the oestrogen response element revealed that ER $\alpha$  had a 2-fold higher potency than ER $\beta$  in mediating E<sub>2</sub> action. Binding studies using <sup>3</sup>H-E<sub>2</sub> revealed comparable ER expression but 4 fold higher binding affinity of E<sub>2</sub> to ER $\alpha$  than to ER $\beta$ . In conclusion, E<sub>2</sub> but not 4-HT suppresses GH signalling in human hepatic tissue. The absence of an effect with 4-HT suggests that oestrogen and SERMs have distinct effect on GH action. ER $\alpha$  is more potent than ER $\beta$  in mediating the E<sub>2</sub> action. (Supported by the SVC Foundation and NHMRC of Australia).

## PREIMPLANTATION ACTIONS OF GROWTH HORMONE BYPASS TYPE 1 IGF RECEPTOR.

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Growth hormone (GH) receptors (GHR) are expressed in ovulated oocytes, zygotes and all preimplantation stages of embryonic development (1). Whilst it is likely that the oocyte GHR are activated by follicular GH, the role of the GHR in preimplantation physiology remains to be elucidated. GH is anabolic in blastocysts (1) and improves morphological development of embryos (2), but is GH hyperplastic? Two further questions arise, firstly since IGF-I is also expressed by the embryo from the 2-cell stage and the type 1 IGF receptor (IGF1R) from the 8-cell stage, does GHR activation lead to signalling through IGF1R? and secondly, does embryonic GH regulate development by an auto/paracrine path? The aim of this study was to investigate if (GH) causes cell proliferation in mouse blastocysts and if this effect is mediated via IGF-I action.

Mouse embryos were cultured in BMOC2 medium with supplements of GH and/or antisera from the 2-cell stage for 48 h and the cell populations of inner cell mass (ICM) and trophectoderm (TE) counted using differential nuclear staining.

In the absence of other additives, anti-GH antiserum reduced blastocyst size by 10-20% ( $P$ <0.01). GH increased total cell number by 20% ( $P$ <0.01) in a bell shaped dose response curve with maximum effect at 1-10 pg/ml, whilst 1 pg GH/ml selectively increased TE by 25% ( $P$ <0.01). Anti-IGF-I receptor antiserum was completely ineffective on this stimulation of TE by GH, but in the absence of GH caused a 20% reduction in ICM size ( $P$ <0.01).

GH caused TE growth via auto/paracrine paths, which operate during preimplantation development. Because of the central part that TE and its derivatives play in implantation, this GH effect may be implicated in implantation and subsequent embryonic development. The results with anti-IGF-I receptor antiserum show that both GH and IGF-I act in an auto/paracrine route to separately stimulate ICM and TE growth and must both be important for optimal blastocyst development.

## ANDROGEN AND INSULIN-LIKE GROWTH FACTOR-1 INTERACTION IN LNCAP CELLS

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Prostate cancer growth results from a deregulation of cell proliferation (mitogenesis) and/or cell death. In this study, to determine the effect of IGF-1 on androgen induced proliferation; we investigated the interaction of Insulin like growth factor-1 (IGF-1) on androgen receptor (AR) activation in the prostate cancer cell line LNCaP. Cell cultures were pretreated with 1 nM 5 $\alpha$ -dihydrotestosterone (DHT) for 48h prior to addition of 10 ng/ml Des (1-3) IGF-1. Whereas cells cultured in 1 nM DHT alone continued to proliferate, addition of Des (1-3) IGF-1 to DHT cultures inhibited proliferation. Moreover, the magnitude of inhibition correlated positively with concentration of Des (1-3) IGF-1 (0-50 ng/ml). AR protein expression was not altered by addition of Des (1-3) IGF-1 to LNCaP cells cultured in DHT. Furthermore, IGF-1 did not inhibit the functional activity of the endogenously expressed mutant AR (877Thr-Ala) on either of two transiently transfected androgen responsive promoters linked to a luciferase reporter gene. Immunoblot analyses demonstrated that, IGF-1 receptor upregulation was evident by 8 h from treatment with 1 nM DHT, and resulted in a two to four fold increase by 24 and 72 h, respectively, over levels in control cultures. IGF-1 receptor upregulation by DHT was abrogated by the anti-androgen Casodex (10 $\mu$ M). DHT treatment however, did not alter the expression of Shc and Grb2, downstream targets of the IGF-1 receptor. Our findings indicate androgen upregulation of the IGF-1 receptor and suggest that activation of the IGF-1 receptor possibly via the Mek-MAPK pathway, may prevent androgen induced proliferation in LNCaP cells.

## REGULATION OF KERATINOCYTE PROLIFERATION AND APOPTOSIS: USE OF ANTISENSE OLIGONUCLEOTIDE INHIBITION TO DEMONSTRATE A DUAL ROLE FOR THE IGF-I RECEPTOR

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Activation of the IGF-I receptor elicits potent mitogenic, anti-apoptotic and transforming signals in many cell types (Baserga, 2000). We have studied the role of the IGF-I receptor in the control of cell proliferation in the human epidermis using antisense oligonucleotide inhibition of receptor expression, and recently demonstrated that IGF-I receptor activation is a rate-limiting step in epidermal hyperproliferation (Wraight et al., 2000). Here we investigate the sequence dependence of target inhibition by our IGF-I receptor oligonucleotides as a prelude to future studies on receptor function in these and other cell types, and also address the hypothesis that IGF-I receptor activation is not only required for keratinocyte proliferation, but is actually an absolute requirement for keratinocyte survival. We transfected human immortalised keratinocytes with C5-propynyl-dU,dC-phosphorothioate antisense oligonucleotide 15mers to the human IGF-I receptor mRNA. We have previously demonstrated up to 80% inhibition of IGF-I receptor expression with one totally homologous oligonucleotide, DT1064 (White et al., 2000). In order to demonstrate the specificity of DT1064, we compared its effects on IGF-I receptor expression to a set of oligonucleotides containing between one and fifteen base mismatches. IGF-I receptor protein levels were measured by immunoblot and mRNA levels by RNase protection assay. We found similar levels of inhibition of expression in HaCaT cells (between 60% and 80% reduction in IGF-I receptor mRNA and protein) for DT1064 and the one- and two-base mismatch oligonucleotides, while oligonucleotides containing four or more mismatches were ineffective. We then examined keratinocyte proliferation and apoptosis levels using a cell counting assay (amido black) and a cell death detection ELISA. Keratinocyte proliferation was completely abolished by DT1064 over a four day period compared to a 2.5 to 3-fold increase in cell number in the presence of 30-100nM control mismatch oligonucleotides. In the presence of serum, DT1064 at 30nM also caused a 175% increase in apoptosis rate when compared with cells transfected with a mismatch control oligonucleotide. These data demonstrate firstly that IGF-I receptor inhibition by DT1064 is sequence dependent, illustrating the potential usefulness of DT1064 as a powerful and specific inhibitor for functional studies on the receptor. Secondly, the data suggest that the IGF-I receptor plays an essential and unique dual role in controlling cell proliferation and apoptosis rates in human keratinocytes, thus identifying a major control point in normal epidermal homeostasis, and suggesting that the IGF-I receptor is a useful cellular target for therapeutic intervention in disease states involving unbalanced proliferation and apoptosis rates, such as psoriasis and skin cancer.

## CASEIN KINASE II SITES IN THE INTRACELLULAR C-TERMINAL DOMAIN OF THE TRH RECEPTOR AND GNRH RECEPTORS CONTRIBUTE TO $\beta$ -ARRESTIN-DEPENDENT INTERNALIZATION

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We have previously shown that the mammalian gonadotropin-releasing hormone receptor (GnRHR), a unique G-protein coupled receptor (GPCR) lacking an intracellular carboxy tail (C-tail), does not follow a  $\beta$ -arrestin-dependent internalization pathway. However, internalization of a chimeric GnRHR with the thyrotropin-releasing hormone receptor (TRHR) C-tail does utilize  $\beta$ -arrestin. Here, we have investigated the sites within the intracellular C-tail domain that are important for conferring  $\beta$ -arrestin-dependent internalization. In contrast to the chimeric GnRHR with a TRHR C-tail, a chimeric GnRHR with the catfish (cf) GnRHR C-tail is not  $\beta$ -arrestin-dependent. Sequence comparisons between these chimeric receptors show three consensus phosphorylation sites for casein kinase II (CKII) in the TRHR C-tail but none in the cfGnRHR C-tail. We thus investigated a role for CKII sites in determining GPCR internalization via  $\beta$ -arrestin. Sequential introduction of three CKII sites into the chimera with the cf C-tail (H354D, A366E, G371D), resulted in a change in the pattern of receptor phosphorylation and  $\beta$ -arrestin-dependence, which only occurred when all three sites were introduced. Conversely, mutation of the putative CKII sites (T365A, T371A, S383A) in the C-tail of a  $\beta$ -arrestin sensitive GPCR, the TRHR, resulted in decreased receptor phosphorylation and a loss of  $\beta$ -arrestin-dependence. Mutation of all three CKII sites was necessary before a loss of  $\beta$ -arrestin-dependence was observed. Visualization of  $\beta$ -arrestin/GFP redistribution using confocal microscopy confirmed a loss or gain of  $\beta$ -arrestin sensitivity for receptor mutants. Internalization of receptors without C-tail CKII sites was promoted by a phosphorylation-independent  $\beta$ -arrestin mutant (R169E), suggesting that these receptors do not contain the necessary phosphorylation sites required for  $\beta$ -arrestin-dependent internalization. Apigenin, a specific CKII inhibitor, blocked the increase in receptor internalization by  $\beta$ -arrestin, thus providing further support for the involvement of CKII. In addition, a GnRHR stop codon mutant with an extended 3' C-tail (ghost tail) is also  $\beta$ -arrestin-independent and does not contain any consensus phosphorylation sites for PKC or CKII. However, internalization of this receptor can be promoted by  $\beta$ -arrestin (R169E) and only the introduction into the ghost tail of three consensus sites for CKII and not PKC, were required for  $\beta$ -arrestin-dependent internalization. This study presents evidence of a novel role for C-tail CKII consensus sites and demonstrates that the addition of a random C-tail sequence, provided it contains CKII sites, can target these GPCRs to the  $\beta$ -arrestin-dependent pathway.

## GROWTH HORMONE-RELEASING PEPTIDE (GHRP-2) ENHANCES INWARD-RECTIFYING POTASSIUM (KIR) CURRENTS IN PRIMARY CULTURED OVINE SOMATOTROPES

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Inward-rectifying potassium (Kir) channels are essential for maintaining the resting membrane potential and responsible for hyperpolarisation-induced K<sup>+</sup> influx in a wide variety of cell types. It was suggested that Kir channels also play an important role in the regulation of GH secretion by somatostatin and synthetic GH secretagogues. GHRP-2 is a potent synthetic peptidergic GH secretagogue, which induced a significant depolarisation in ovine somatotropes. The mechanism for such a depolarisation has not yet been clarified. In this study, we characterised the Kir currents in immunocytochemically identified somatotropes and investigated the effect of GHRP-2 on this current by using nystatin-perforated whole-cell patch clamp techniques. Ovine pituitary cells were obtained by collagenase dissociation, followed by the percoll-gradient separation to enrich somatotropes to 60-80% of total cells. Using a low Ca<sup>2+</sup> (0.5 mM) bath solution containing 5 mM K<sup>+</sup> and 1  $\mu$ M TTX and high K<sup>+</sup> (140 mM) pipette solution, ovine somatotropes exhibited inward rectifier K<sup>+</sup> currents (Kir) when cells were held at -50 mV and stepped from -160 mV to -40 mV with 10 mV interval (200 msec). The Kir currents were relatively small (less than 50 pA) and rapidly activated. The delayed part of Kir currents became significant and increased dramatically (up to 4-fold increase, the transient part of the current was only increased about 20%) by extracellular administration of high K<sup>+</sup> concentration (25 mM). This phenomenon was only observed in immunocytochemically identified somatotropes but not in other type of endocrine cells (negatively stained with anti-GH antibodies) in the same culture condition. Similar observations were obtained in a rat somatotrope cell line, GC cells with overexpression of GH-releasing hormone receptors. In addition, the Kir currents in somatotropes were blocked in a dose dependent manner by external Ba<sup>2+</sup> and Cs<sup>+</sup>, which confirmed the biophysical character of the Kir currents. Application of GHRP-2 (100 nM) onto the recorded cells reversibly reduced the Kir currents by about 25%. The response occurred in 1-2 min after application of GHRP-2 and reached maximum inhibition within 3-5 min. These data suggest that ovine somatotropes and rat GC cells have the Kir currents which responding to high extracellular K<sup>+</sup> condition. Such currents may contribute to the maintenance of membrane resting potential and the inhibition of the Kir currents by GHRP-2 may play an important role in the depolarisation, which leads to the increase in [Ca<sup>2+</sup>]<sub>i</sub> and GH secretion. Signalling system involved in this inhibitory effect of GHRP-2 on the Kir currents is under investigation. (Supported by NHMRC and R. Xu is a recipient of Monash Graduate Scholarship).

## CHARACTERIZATION OF THE MECHANISMS OF AUTOCRINE INDUCED SIGNAL TRANSDUCTION IN THE 2-CELL EMBRYO.

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Embryo-derived platelet-activating factor (PAF) induces transient increases in intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) in the zygote and 2-cell stage mouse embryo. These  $[Ca^{2+}]_i$  transients are essential for normal embryo development and requires the influx of extracellular calcium (1). The aim of the current study was to characterize the manner in which these transients were generated by PAF. Fresh 2-cell embryos were collected from superovulated mice. They were labeled with fura-2 AM and changes in  $[Ca^{2+}]_i$  in response to PAF (200 ng/ml) were assessed by the ratiometric imaging of Fura-2 at 340 and 380 nm. Normal PAF-induced  $[Ca^{2+}]_i$  transients were receptor-dependent but occurred in embryos in which the known PAF-receptor had been deleted by homologous recombination, implicating the actions of a novel PAF-receptor in the embryo. The absence of the receptor was confirmed by immunolocalization. We confirmed that the calcium transient in PAF-receptor KO mice was entirely dependent upon extracellular calcium. Nifedipine blocked the ( $[Ca^{2+}]_i$ ) transient while the L-type channel agonist BAY K 8644 mimicked the response. Other L-type channel antagonists, diltiazem and verapamil, caused significant inhibition of the transient. RT-PCR showed that the 2-cell embryo expressed alpha-C but not the alpha-D L-type channel, and expression at the protein level was confirmed by immunolocalization. Inhibitors of the other major voltage-gated channel classes were without effect and it was shown that the calcium influx was not voltage-dependent. Inhibition of store-operated channels (SOC) did not affect the response to PAF and quantification of SOC predicted that there was insufficient SOC activity within the 2-cell embryo to generate the transients. These results suggest that the ( $[Ca^{2+}]_i$ ) transient required the action of an L-type channel, but did not require voltage gating. Activation of the  $[Ca^{2+}]_i$  transient was blocked by a tyrosine kinase inhibitor (100 microM genistein) and by a  $G_i$  inhibitor and  $G_s$  activator (100ng/ml Pertussis toxin, and 20 microg/ml Cholera toxin). The results show that PAF-induced intracellular  $Ca^{2+}$  transients in the 2-cell embryo are due to the actions of a novel PAF-receptor that activates an L-type calcium channel in a voltage-independent fashion. The regulation of the channel by this receptor is tyrosine kinase and  $G_i$  dependent with partial negative regulation by  $G_s$ . This receptor-dependent, voltage-independent activation of the L-type channel may form a novel mechanism of signal transduction in the early embryo.

## HOMO-OLIGOMERIZATION OF THE THYROTROPIN RELEASING HORMONE (TRH) AND GONADOTROPIN RELEASING HORMONE (GnRH) RECEPTORS : DETECTION USING BRET

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G protein coupled receptors (GPCRs) have traditionally been thought to function as monomeric units, however, a growing body of biochemical and functional evidence supports the existence of homo- and hetero-dimerization amongst receptors and a critical role for dimerization in many aspects of receptor function. The thyrotropin releasing hormone receptor (TRHR) and gonadotropin releasing hormone receptor (GnRHR) are GPCRs which are both expressed in the anterior pituitary. Although the trafficking and signalling of the TRHR and GnRHR are well characterised, the role of receptor-receptor interactions in receptor function is unknown. We have employed the newly developed bioluminescence resonance energy transfer (BRET) technique to assess whether the TRHR and GnRHR are capable of forming homo-oligomers in living cells. The formation of constitutive TRHR oligomers was demonstrated in COS 1 cells by a transfer of energy between co-transfected TRHR molecules fused to either donor (Renilla luciferase) or acceptor (EYFP) molecules. Agonist-stimulation led to a further dose-dependent increase in the amount of energy transfer, suggesting that receptor oligomerization plays a role in signal transduction. This TRH-induced BRET was due to an interaction between receptors at the cell surface and not due to internalization. In addition, an inverse agonist, midazolam, was shown to block the agonist-induced TRHR BRET, but not to affect the formation of pre-existing TRHR oligomers. The use of tagged  $\beta_2$ adrenergic or GnRH receptors and over-expression of untagged TRHR, demonstrated that the interaction between TRHRs was specific. In addition, we were unable to detect any interaction between the TRHR and GnRHR. The technique was also applied to the GnRHR, demonstrating that it can undergo oligomerization in response to an agonist but not antagonist. We have used the BRET technique to demonstrate that homodimers form for both the GnRHR and TRHR, providing further evidence that homodimerization is a general phenomenon amongst GPCRs and plays an important role in receptor function.

## INTERDOMAIN INTERACTIONS IN THE MINERALOCORTICOID RECEPTOR

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The actions of the steroid hormone aldosterone in sodium reabsorption and the cardiovascular system are mediated by the mineralocorticoid receptor (MR). The MR is a modular protein consisting of three major functional domains: the N-terminal domain, the DNA-binding domain (DBD) and the ligand-binding domain (LBD). The DBD and LBD are separated by the hinge region, the function of which is unknown. As with other steroid hormone receptors (SHRs) each domain of the MR can function independently. However there is mounting evidence in SHRs for "cross-talk" between domains which is important for receptor activation. This is best described for the androgen receptor (AR), where interactions between the N-terminal and ligand binding domains are important for the stability of ligand binding; mutations that disrupt this interaction can cause androgen insensitivity syndrome. We have examined the potential for interaction between the N-terminal domain and the C-terminal region (hinge and LBD) of the MR using the mammalian-2-hybrid assay. The MR C-terminal region was fused to the Gal4 DBD (GAL4-MR C) and the N-terminal domain was fused to the VP16 transactivation domain (VP16-MR NT). The chimeras were transfected into COS-1 cells together with a Gal4-responsive luciferase reporter gene. An increase in luciferase expression is observed if the two domains of the MR interact. GAL4-MR C displays ligand-dependent, VP16-independent transcriptional activity in the assay. To remove this complication a mutation was created at a conserved glutamine (E962A) that has been shown in other SHRs to be critical for LBD-mediated transactivation. This mutant, GAL4-MR C(E962A), displays no baseline activity in the system. In the mammalian-2-hybrid assay both GAL4-MR C and GAL4-MR C(E962A) interact with VP16-MR NT in the presence of aldosterone. The interaction is concentration-dependent with an EC<sub>50</sub> of 1-3 nM. To assess the importance of this interaction it was compared directly to the well-characterised AR N/C-interaction. The magnitude of the interaction between GAL4-MR C/GAL4-MR C(E962A) and VP16-MR NT is similar to that between GAL4-AR LBD and VP16-AR NT, suggesting that the MR N/C-interaction is important to receptor function. There is no interaction between GAL4-MR C(E962A) and VP16-MR NT in the presence of the MR antagonist spironolactone. Spironolactone also inhibits aldosterone-mediated association of the two domains in a concentration-dependent manner. These results suggest that the N/C-interaction is dependent on the conformation of the MR LBD, as aldosterone and spironolactone are known to induce different conformations in this domain. The N/C-interaction is specific, as GAL4-MR C(E962A) does not interact with either the AR or glucocorticoid receptor N-terminal domains in the presence of aldosterone. We have demonstrated for the first time interdomain interactions in the MR. This N/C-interaction may be important for MR homodimerisation and may have other effects on receptor function.

## A LEUCINE-RICH DOMAIN INCORPORATING THE LXXLL MOTIF IS AN INTEGRAL PART OF A COMPOSITE BINDING SITE IN HEAT SHOCK PROTEIN 90β FOR STEROID RECEPTOR-ASSOCIATED IMMUNOPHILINS.

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Steroid receptor complexes contain heat shock protein 90 (hsp90) and one of a number of immunophilins: cyclophilin 40 (CyP40), FKBP51, FKBP52 and PP5 which appear to modulate receptor function. Immunophilins compete via conserved tetratricopeptide repeat (TPR) domains for a TPR-acceptor site located in the C-terminal domain of hsp90. Essential for this interaction is the strongly conserved MEEVD sequence at the extreme C-terminus of hsp90. This peptide is accommodated within a binding groove assembled from the 3 unit TPR domain of CyP40. Evidence suggests that regions N-terminal to this MEEVD motif may also contribute to hsp90-TPR protein interaction. A leucine-rich hydrophobic  $\alpha$ -helical microdomain, incorporating residues 649-670, is present upstream of the MEEVD motif. This microdomain includes an LXXLL sequence motif similar to those recently shown to mediate coactivator interaction with nuclear receptors. Modelling of this  $\alpha$ -helical peptide onto the CyP40 TPR domain suggests that basic residues preceding the LXXLL motif may facilitate docking of this microdomain onto the CyP40 surface. The aim of the present study was to perform a mutational analysis of selected residues within this microdomain to investigate their role in hsp90-CyP40 interaction.

Using site-directed mutagenesis, we introduced single point alanine substitutions for lysine and large hydrophobic residues within this microdomain of human hsp90 $\beta$ , the C-terminal domain (520-724) of which was cloned into the yeast 2-hybrid prey vector pVP16. Mutants were cotransfected into yeast strain L40 with a bait pBTM116 plasmid containing a LexA DNA-binding domain fused to a wild-type human CyP40 C-terminal sequence (185-370) and analysed for binding using a  $\beta$ -galactosidase assay. Double mutations at L657/L658 and L663/L664 and a single substitution at L670 dramatically inhibited hsp90-CyP40 binding. Mutation of L654, at the start of the LXXLL motif, and of residues F659 and F668 had little impact on this interaction. Our study provides evidence that leucine residues within the hydrophobic microdomain play a key role in CyP40 recognition. We are currently determining whether single, rather than double, leucine mutations and upstream lysine substitutions within this microdomain can disrupt hsp90-CyP40 binding.

## FUNCTIONAL INTERACTION BETWEEN ESTROGEN RECEPTOR $\alpha$ AND A NOVEL COACTIVATOR VIA ACTIVATION FUNCTION-1

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The estrogen receptor (ER) is a ligand-dependent transcription factor. The ER activates target genes via two transactivation functions: a ligand dependent AF2, which is highly conserved across the nuclear receptor superfamily, and a ligand-independent, receptor-specific, transactivation domain AF1. While pure agonists (such as estrogen [E2]) activate both AF1 and AF2, partial agonists (SERMS) such as tamoxifen inactivate AF2 and act via AF1 alone. Transcriptional activation is modulated by proteins which bind to the receptor to enhance (coactivators) or inhibit (corepressors) transcription. Major coactivator families identified to date interact with and activate nuclear receptors via AF2. These studies aimed to instead identify AF1-specific coactivators which might contribute to the residual activity of the ER in the presence of tamoxifen (TAM) or other SERMS.

Putative ER-interacting proteins were isolated from a mouse embryo cDNA library by 2-hybrid screening in PJ694A yeast cells using full-length, mutant ER $\square$ , linked to the Gal4-DNA binding domain (DBD), as bait. This mutant receptor (ER-AF1) contains two inactivating mutations in AF2, but retains AF-1, as well as ligand binding, activity. 10<sup>7</sup> library clones were screened, using three independent reporter assays (ADE and HIS growth assays and a  $\beta$ -galactosidase ( $\beta$ -gal) assay). 300 putative interactants were initially identified. Next, false positive interactants were further excluded by remating the isolated library clones with a series of bait constructs, including the bait vector alone, wildtype ER, and other AF2 mutants. The remaining putative ligand-dependent and AF1-dependent interactants were isolated and sequenced.

One of these putative coactivators is highly homologous (e10<sup>-143</sup>) to a 807 amino acid region within the enzyme carbamoyl phosphate synthetase II/aspartate transcarboxylase/ dihydroorotase (CAD). CAD is the rate limiting enzyme in pyrimidine synthesis, and its activity is upregulated up to 8-fold in some human cancers, including breast cancer. Binding of the CAD807 clone to the full-length ER was estrogen-dependent [ $\beta$ gal:ER-AF1=2.2 $\pm$ 0.2(no hormone[NH]); 1.7 $\pm$ 0.3(10 nM E2); CAD=0 (NH); 0.6 $\pm$  0.5 (E2);ER-AF1+CAD=1.8  $\pm$ 0.3(NH); 21 $\pm$  2(E2)]. This clone binds in a ligand-dependent fashion to two different mutant receptors, each containing an intact AF1 but with mutations in AF2, as well as to the wildtype receptor. We next demonstrated that CAD and ER $\alpha$  also interact *in vitro*, using *in vitro* translated CAD and a glutathioneS-transferase/ER fusion protein (GST pulldown) or DNA bound ER (ABCD) assays. Next, flag-tagged CAD was transfected into Cos1 cells in the absence and presence of the ER, immunolabelled, and analysed using a Zeiss confocal microscope. ER $\alpha$  alters the intracellular distribution of CAD in a ligand-dependent as well as a ligand-independent fashion, demonstrating that these proteins also interact *in vivo* in mammalian cells (%nuclear CAD= 2.6% (no ER); 47% (ER); 84% (ER+E2). Finally, increasing amounts of CAD were co-transfected with the ER and a luciferase reporter. CAD enhances transcriptional activation by the ER in a ligand-dependent, as well as a ligand-independent, fashion (Relative Activity: No Hormone: ER 18.3 $\pm$ 3.1, ER+40ngCAD 709 $\pm$ 121; E2: ER 100, ER +CAD 1179 $\pm$ 239; TAM: ER 8.4 $\pm$ 2.5; ER+CAD: 239 $\pm$ 45).

In summary, CAD interacts with the ER *in vitro* as well as *in vivo* in yeast and in mammalian cells. This interaction is AF1 dependent. Consistent with this, CAD enhances ER-mediated transcriptional activation. These results suggest that CAD may act as a bona fide coactivator of the ER, and might play a role in regulating ER action in disease states where its activity is altered.

## PRIMARY HYPOTHYROIDISM PRESENTING AS A PITUITARY MASS

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A 16 yo female presented to her GP with lethargy, ataxia, myalgia and fatigue for 1 year. Initial investigations showed hyperprolactinaemia (52.6 ng/ml; N < 25.0), elevated TSH (79.8 mU/L; N 0.4-4.7), and CT scan with enlargement of the pituitary fossa due to a 13x11 mm mass reported as a pituitary macroadenoma. She was referred for neurosurgical assessment. Clinical evaluation revealed a 1 year history of dry skin, cold extremities and fine scalp hair. Deterioration in sporting performance had occurred over 3-4 y. Menarche at 12y was followed by irregular cycles with regular periods for only 5 months at age 14y with subsequent oligomenorrhoea and 5 months' amenorrhoea. Height was below median and she admitted to difficulty following the thread of conversations. One grandmother had primary hypothyroidism and one grandfather had a pituitary tumour. Height was 1.60m, weight 59kg, she had severely slowed reflexes, percussion myotonia and profuse lanugo hair. MRI confirmed uniform enhancement of an enlarged pituitary with an 8mm 'circumscribed nodule' encroaching on the optic chiasm and elevating the R optic nerve. Visual fields were normal, FT4 <5 pmol/L, TSH 329 (0.5-5.0), CK 646 U/L (<200), alpha-glycoprotein subunit (AGS) 6.39 IU/L (0.05-0.55), FSH 5.2 U/L (0.8-10.0), LH <0.5 U/L(0.8-15.0), oestradiol 418pmol/L (follicular phase 110-360), 2 pm cortisol 329nmol/L (120-400), GH 2.6 mU/L. A clinical diagnosis of primary hypothyroidism with secondary anterior pituitary hyperplasia was made and thyroxine 50mcg daily commenced with cortisone supplements. MRI was repeated at 5 weeks with 2mm reduction in height and reduced mass effect on optic chiasm. Thyroid function normalized over 8 months with final thyroxine dose 150 mcg daily. At 10 months, weight 54kg, periods were normal, Prolactin 7.5, AGS 0.36. MRI showed virtually normal pituitary size. This case highlights a rare presentation of hypothyroidism requiring recognition to avoid unnecessary surgery.

## HYPOPITUITARISM WITH A HYPOPHYSEAL MASS IN ASSOCIATION WITH GRAVES' DISEASE

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Mrs L, a 42-year-old woman was admitted under the care of a neurologist for investigation of headaches. The headaches had been present for 2 months and were frontal, moderately severe, and intermittently associated with vomiting and photophobia. The patient gave a history of 2 months of amenorrhoea. An endocrine consultation was requested.

Mrs L gave an additional history of several kilograms of recent weight loss. Examination showed a mildly distressed woman of Asian ethnic origin. The blood pressure was 120/70, with a postural drop of 25 mmHg. Pulse rate was 95 beats per minute. The patient was afebrile. A fine resting tremor was present, accompanied by mild proximal muscle weakness. Pupillary reactions, visual fields, fundoscopy and extra-ocular movements were normal. The patient did not have proptosis, but lid retraction and lid lag were present. A diffuse goitre was present, with a bruit on auscultation. There was no abnormal skin pigmentation present. There were signs of recent weight loss.

Investigations showed low oestradiol (<37pmol/L), and low luteinizing hormone (0.1mIU/ml). FreeT4 was 24.9 (10-23.2), and thyroid stimulating hormone (TSH) was <0.01. Thyrotropin releasing hormone stimulation test showed no rise in TSH. Prolactin was raised at 3040 (<440 IU/ml). Cortisol was low at 36nmol/L, accompanied by an undetectable adrenocorticotropic hormone. A short synacthen test was abnormal with baseline of 76, and peak response of 385. Thyroid scan showed uniform increased uptake consistent with Graves' disease. Magnetic resonance imaging of the pituitary gland showed a uniformly enhancing mass in the pituitary stalk with some superior displacement of the optic chiasm.

Mrs L was treated with intravenous hydrocortisone, followed by oral hydrocortisone. Neomercazole and propranolol were commenced to treat thyrotoxicosis. Bromocriptine was commenced. Subsequently Mrs L developed symptoms of polyuria and polydipsia, and underwent a water deprivation test, which was positive, and showed a rapid response to intranasal desmopressin. After one month, hormone replacement therapy was commenced.

Learning points to be reviewed in this case are (I) the differential diagnosis of the pituitary mass, (II) the association between hypophyseal and thyroid disease, (III) whether biopsy is warranted, and (IV) the prognosis for recovery of pituitary function and (V) unmasking of diabetes insipidus following treatment of hypoadrenalism.

## PLASMA CELL GRANULOMA OF THE HYPOTHALAMUS – AN UNUSUAL CAUSE OF PROGRESSIVE HYPOPITUITARISM

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**Presentation:** A 31 year old Samoan woman presented with an 11 month history of secondary amenorrhoea, polyuria, polydipsia, weight gain and lethargy. In retrospect, mild headaches were present at the initial onset of symptoms. Examination documented generalised obesity with a BMI of 35, expressible galactorrhoea and normal visual fields. Investigations revealed hyperprolactinaemia (prolactin 3433 mIU/L (n 50-550)), low gonadotropins and oestradiol. 24 hour urine volume of 10.9 L was consistent with diabetes insipidus. Thyroid function, cortisol secretion and IGF1 were normal. ESR was slightly raised at 40 mm/hr and serum immunoglobulins showed a polyclonal increase in IgA and IgG. MRI demonstrated diffuse enhancement of the hypothalamus and proximal stalk, with enlargement of the optic tracts, consistent with an infiltrative process. Further investigations, including lumbar puncture, looking for an inflammatory cause such as infection or granulomatous disease were negative. Treatment with carbegoline and DDAVP were instituted with plans for possible biopsy.

**Progress:** Three weeks later TSH deficiency was diagnosed on the basis of symptoms, low FT4 (9 (n 11-24)) and TSH (0.82 (n 0.4-4)). Plasma cortisol at 10am was normal at 331 nmol/L. Thyroxine was commenced. Six weeks later further symptoms were associated with ACTH deficiency (9am cortisol of 96 nmol/L) and hydrocortisone was commenced. Repeat visual fields at 8 weeks showed a superior temporal defect in the left eye and MRI scanning showed slight enlargement of the lesion with reduction in volume of the anterior pituitary. The lesion was biopsied via a right pterional craniotomy. Macroscopically both cranial nerves and the optic chiasm were twice normal thickness. A grey, homogeneous, firm, relatively avascular, discrete lesion was seen and two small fragments excised. Histology showed vascular granulation tissue with a moderately dense infiltrate of lymphocytes, plasma cells and monocyte-macrophages. Numerous Russell bodies were present. No granulomas or organisms were seen. Immunohistochemistry confirmed the polyclonal nature of the plasma cells.

**Discussion:** Plasma cell granulomas are reactive, tumour-like, non-neoplastic lesions, sometimes also referred to as "inflammatory pseudo tumours". An admixture of plasma cells, lymphocytes and histiocytes is seen with a striking predominance of plasma cells producing polyclonal immunoglobulins. These lesions commonly occur in the lungs and upper respiratory tract and are rarely seen on other organs, including the liver, spleen, orbit, kidney, thyroid and heart. Plasma cell granulomas of the central nervous system are extremely rare with only 10 cases reported in the literature. Several cases have presented with visual failure and/or diabetes insipidus. Primary surgical excision is advocated although responses to irradiation and glucocorticoids are recorded. Prognosis appears favourable, with the only documented death resulting from a neurosurgical complication. Since surgical excision is technically not feasible in our patient, she is currently receiving a trial of high dose glucocorticoids with a view to future irradiation. **Conclusion:** Plasma cell granuloma is a very rare cause of hypopituitarism and emphasises the importance of biopsy in the diagnosis of infiltrative lesions.

## MCCUNE ALBRIGHT SYNDROME IN A CHILD, COMPLICATED BY ACROMEGALY AND PRECOCIOUS PUBERTY

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McCune Albright syndrome was diagnosed at age 3 years in a male, who presented with a lower limb fracture and was found to have large café au lait markings. (Mutation 601 C>T)

He has extensive polyostotic fibrous dysplasia and has been treated with pamidronate for 4 years, with control of bone pain, improved quality of life and reduced fracture rate. Growth has been monitored at 4–6 monthly intervals with visual fields checked annually from age 3. Gonadotrophin independent precocious puberty occurred at age 7 but was only slowly progressive and required no treatment. Bone age was 9 at 7.5 years.

Between age 8 and 8.5 years, he had an increasing growth velocity with onset of a "square" jaw, widening of spaces between teeth, marked acral enlargement, sweating and facial acne but no change in pubertal status (testes 6ml, pubic hair Tanner stage II). Binasal field loss was present. MRI demonstrated a bulging pituitary gland typical of adolescence, with no discrete tumour, adjacent to but not elevating the optic chiasm. A markedly thickened calvarium and sphenoid were seen (4 cm+) on skull Xray. Bone age advanced from 9 to 13 years in one year.

Basal growth hormone (GH) was 78mU/l, rising to 100mU/l with glucose tolerance test, basal IGF1 2.4IU/L, FSH <0.1mU/l, LH 0.1mU/L, testosterone <1.4nmol/l.

Treatment with octreotide LAR, flutamide and testolactone has resulted in control of GH (12mU/L) and precocious puberty (testes 3ml, decrease in pubic hair), slowing of growth, return to normal of jaw size and tooth spacing, sweating, acne and facial appearance and restoration of full visual fields.

Response has demonstrated short term efficacy of medical treatment in a very difficult case and a remarkable capacity for facial remodelling in a child with acromegaly. Visual field change is attributed to reduction in soft tissue swelling around the optic foramina.

Future issues concern long term control of GH secretion, the impracticability of surgery in this patient and radiotherapy concerns in a young child.

## A FUNCTIONAL DELETION OF THE CALCIUM SENSING RECEPTOR IS PREDICTED BY HETEROZYGOUS TRUNCATION MUTATIONS PRESENT IN AN INFANT WITH NEONATAL SEVERE HYPERPARATHYROIDISM.

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The calcium-sensing receptor (CaR), a G-protein coupled receptor with a classic seven membrane-spanning motif, plays an integral role in regulating extracellular calcium concentrations. The activation of the human CaR induces a signalling pathway that results in a decrease in parathyroid hormone (PTH) secretion in parathyroid cells and inhibits the reabsorption of Ca<sup>2+</sup> in kidneys. Familial hypocalcaemic hypercalcaemia (FHH) is associated with heterozygous inactivating mutations in the CaR. Neonatal severe hyperparathyroidism (NSHPT) occurs when there are inactivating mutations in both CaR alleles. FHH is characterised by lifelong moderate, but generally asymptomatic hypercalcaemia but NSHPT can be life threatening without parathyroidectomy. We report a case of NSHPT in a five-month old male infant with a history of failure to thrive, hypotonia and developmental delay. Biochemical investigation revealed hypercalcaemia, hypocalcauria and hypersecretion of PTH. Skeletal X-rays showed changes consistent with hyperparathyroidism. After initial management to reduce hypercalcaemia, total parathyroidectomy was performed. Post-operatively, the patient continues to make excellent developmental progress. Family history revealed a large number of maternal relatives with known hypercalcaemia consistent with FHH. There was no history of consanguinity in the parents of the proband. DNA sequence analysis of the CaR's coding region revealed the heterozygous nonsense mutations G94Stop and R648Stop. The mutations abolished and introduced restriction enzyme recognition sites for XcmI and DdeI, respectively. Digestion with these enzymes confirmed both mutations in the infant, but only the R648Stop mutation was present in the proband's hypercalcaemic mother and other affected maternal relatives. Analysis of 50 unrelated normal subjects revealed the absence of both mutations. The R648Stop mutation, located immediately before the second transmembrane domain, would produce a truncated CaR lacking domains critical for ligand binding and G protein-coupled signalling. The G94Stop mutation, near the N-terminus in the extracellular domain, would yield a severely truncated receptor that would not be anchored in the cell membrane. Together these mutations potentially correspond to a functional deletion of the CaR in the NSHPT patient. CaR mutants, corresponding to these mutations, created by site-directed mutagenesis have been cassetted with and without a FLAG epitope into wild-type CaR<sub>pcDNA1</sub> recombinant vector for expression in HEK293 cells. Constructs with the FLAG epitope will be used to determine the expression of wild-type and truncated receptors in HEK293 cells by Western analysis. An assay measuring phosphoinositide hydrolysis, a signalling event downstream of CaR activation is currently being used to determine the functional effects of each mutation on the CaR.



## PROSTAGLANDIN E<sub>2</sub> CAN DIRECTLY STIMULATE CORTISOL PRODUCTION FROM THE OVINE FETAL ADRENAL GLAND *IN VIVO*

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In the late gestation ovine fetus there is a large increase in plasma cortisol concentrations that drives maturational events and labour. Increases in ACTH secretion or activity appear insufficient to fully explain the late gestation increase in cortisol secretion, and we have recently demonstrated that no increase in plasma ACTH concentrations is necessary to reproduce normal, increasing cortisol concentrations in the late-gestation fetus (1). These observations have led us to hypothesise that other factor(s), in particular prostaglandin (PG) E<sub>2</sub>, could also act at the fetal adrenal to directly cause the secretion of cortisol and potentially play a role in the prepartum cortisol surge. In support of our hypothesis we have recently demonstrated that PGE<sub>2</sub> is capable, *in vitro*, of stimulating cortisol secretion from isolated adrenal slices obtained from late-gestation ovine fetuses (2). The aim of the present study was to examine this hypothesis *in vivo*.

Fetuses were allocated into one of two groups: intact (INT) fetuses infused with saline (INT/SAL), or hypophysectomised (HX) fetuses chronically maintained with a low-dose, infusion of ACTH that achieved constant plasma ACTH concentrations (HX/ACTH). The ACTH infusion maintains the integrity of the fetal adrenal cortex. Each fetus received a CRF/AVP challenge at 130 d gestational age (GA) consisting of a bolus dose of CRF (1 µg) + AVP (0.2 µg) to examine responsiveness of the fetal pituitary gland. At 134 d GA a hypoglycemia challenge was also performed by injecting the ewe with 2 IU insulin/kg body weight to examine the effects of a physiological stressor on the fetal hypothalamo-pituitary-adrenal (HPA) axes of these fetuses. These INT/SAL and HX/ACTH groups were then further divided with each half receiving either a 24 hour (h) infusion of saline (1 ml/h) or PGE<sub>2</sub> (1 µg/kg/min) at 140 days gestational age to determine the adrenal cortisol response.

The CRF/AVP and hypoglycemia challenges both causes significant increases in plasma cortisol concentrations in the INT/SAL but not the HX/ACTH fetuses, indicating the completeness of the HX procedure and the lack of responsiveness of the HPA axis in HX animals to a stressor that is integrated *via* central nervous systems sites. In contrast, the 24 h PGE<sub>2</sub> infusion significantly increased plasma cortisol concentrations in both the INT/SAL and HX/ACTH groups; the 24 h infusion of saline was without effect on cortisol concentrations in either group.

These observations demonstrate that PGE<sub>2</sub> can directly stimulate the fetal adrenal gland *in vivo* to cause cortisol secretion, and that an intact hypothalamo-pituitary unit is not necessary for this effect to occur. Furthermore, the observations suggest that PGE<sub>2</sub> may play a role in the prepartum cortisol surge.

## IDENTIFICATION AND CLONING OF THE VARIANT TRANSCRIPTS OF THE WALLABY GONADOTROPHIN-RELEASING HORMONE RECEPTOR

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Gonadotrophin-releasing hormone (GnRH) plays a critical role in the regulation of the hypothalamic-pituitary-gonadal axis. It binds to the GnRH receptor (GnRH-R) in the anterior pituitary to regulate the secretion of gonadotropins. The aim of this study is to examine the molecular structure and expression of GnRH-R in the tammar wallaby, in order to provide fundamental knowledge about marsupial GnRH and its regulation of the hypothalamic-pituitary-gonadal axis. We have cloned and partially characterised the wallaby GnRH-R from the pituitary and the testis. Like other mammalian GnRH-R, the wallaby GnRH-R also contains 3 exons and 2 introns. Using RT-PCR and the wallaby pituitary total RNA as a template, we isolated 2 variant transcripts. The first variant transcript (GnRH-RA1) was characterised by a 291 bp deletion from nucleotide positions 232 to 522, and this deletion is located in the exon 1. The GnRH-RA1 transcript was generated by alternative splicing with accepting an alternative splice donor site in the exon 1. Interestingly, the deletion does not cause a frame shift of the open reading frame. Translation from the putative start-site yields a protein with 231 amino acid. The second variant transcript (GnRH-RA2) was also generated by alternative splicing and was characterised by a 220 bp deletion from nucleotide positions 523 to 742, corresponding to exon 2. This deletion causes a shift in the reading frame, and translation was terminated after the third amino acid down stream from the splice junction. It encodes a protein of only 177 amino acids. Furthermore, we have also studied the expression of these variant transcripts in the testis using RT-PCR, and the GnRH-RA1 transcript was found. However, no GnRH-RA2 transcript was detected in the testis. The expression of the GnRH-RA2 transcript appears to be pituitary specific.

## UTERINE DISTENSION IS A KEY FACTOR IN THE REGULATION OF OXYTOCIN RECEPTORS IN THE TAMMAR WALLABY.

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Oxytocin (OT) has a wide range of physiological actions in mammals, including contraction of uterine myometrial cells to expel the fetus during parturition. Female marsupials have two separate uteri and in monovular species, such as the tamarin wallaby, one uterus is gravid with the single fetus whereas the contralateral uterus is nongravid. In the pregnant tamarin, there is a marked increase in myometrial OT receptors (OTRs) on Day 23 of the 26-day gestation but in the gravid uterus only. The aim of this study was to investigate whether or not distension of the gravid uterus is responsible for the upregulation in OTRs. The first experiment involved surgical removal of the fetus on Day 20 of gestation. There were three surgical groups: i) fetectomy, including placenta and uterine fluid; n=4 ii) fetectomy and placenta only; n=4 iii) sham operated controls; n=3. Three days after surgery, the animals were euthanized, myometrial tissues were collected and OTR concentrations measured in both uteri using a radioreceptor assay with <sup>125</sup>I-OTA as the labeled ligand. In all surgical groups there was a significant (p<0.05: paired t-test) increase in OTRs observed in the gravid uterus compared with the nongravid. Reducing distension in the gravid uterus via removal of the fetoplacental unit and yolk sac fluid (YSF) resulted in a significant (p=0.03: Mann-Whitney U test) decrease in myometrial OTRs (334.6 ± 60.3 fmol/mg protein) compared with controls (584.6 ± 28.5 fmol/mg protein). In contrast, there was no significant difference in myometrial OTR concentrations in the gravid uterus of sham operated controls and animals in which uterine distension was maintained without the fetoplacental unit (454.6 ± 112.6 fmol/mg protein). OTRs in the nongravid uterus were not significantly affected by surgery. We then examined OTR concentrations in the myometrium of animals that appeared pregnant with a large, distended uterus (n=6). However, these uteri lacked a normal fetus or vascular placenta. In all animals, OTR concentrations in the distended uterus (479 ± 103.3 fmol/mg protein) were significantly (p=0.04: paired t-test) higher than the non-distended uterus (171.4 ± 52.2 fmol/mg protein). The data from these experiments demonstrate that distension of the gravid uterus may affect OTR upregulation in the myometrium of pregnant tamarin wallabies.

## THE EFFECT OF A CRH ANTAGONIST ON TADPOLE GROWTH AND DEVELOPMENT DURING METAMORPHOSIS

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There is evidence that CRH is involved in the onset of metamorphosis in tadpoles. It has been suggested that the involvement of CRH may allow tadpoles to respond to external stressors such as pond desiccation by undergoing an early metamorphosis, allowing them the means to escape the adverse condition and find a new habitat. Treatment with the CRH receptor antagonist  $\alpha$ -helical CRH<sub>9,41</sub> has been shown to delay metamorphosis in *Scaphiopus hammondi* tadpoles. This antagonist acts by blocking both type I and II CRH receptors. During the stress response, CRH is thought to act via its type I receptor.

To determine whether the effect of CRH is indeed related to the stress response, we treated tadpoles with the CRH type I receptor antagonist, antalarmin. Tadpoles were given antalarmin mixed in with their food at concentrations of 0, 5 and 25mg per litre of experimental pond water. Tadpoles were treated with antalarmin long- or short-term. Long-term treated tadpoles received antalarmin from when they first became free-swimming and free-feeding until the end of the experiment (metamorphosis of controls). Short-term treatment lasted for 14 days, during late premetamorphosis, when hind limb bud development was beginning. The long-term treatment was carried out on two species from the Myobatrachidae, one of the 2 major families of frogs endemic to Australia. These were *Limnodynastes tasmaniensis* and *Limnodynastes peronii*. Short-term treatment was carried out on *L. tasmaniensis*.

In all experiments, treatment with antalarmin decreased food intake in a dose-dependent manner. While tadpoles ate sufficiently to survive the experiments, development of the long-term treated tadpoles was curbed greatly in both species. Growth also was inhibited in *L. tasmaniensis* tadpoles. This decreased appetite was unexpected as CRH is raised during anorexia, where appetite is decreased, so treatment with a CRH antagonist was expected to increase appetite. Normal feeding resumed after treatment had ceased in the short-term treated tadpoles, as did growth and development. CRH levels were increased in a dose-dependent manner at similar developmental stages in *L. tasmaniensis* tadpoles treated long-term with antalarmin. Increased brain CRH was also seen in *L. peronii* tadpoles at the higher antalarmin dose.

*Limnodynastes tasmaniensis* tadpoles treated short-term with the high concentration of antalarmin showed a decrease in brain CRH at premetamorphosis, during very early hind limb bud development, with a return to the same level as controls by prometamorphosis (early metamorphosis, up to but not including forelimb emergence). These studies suggest that type I CRH receptor may be involved in the regulation of appetite in metamorphosing tadpoles.

## NOVEL INTERACTIONS BETWEEN REGULATORY ELEMENTS OF THE CRH GENE.

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CRH production in the hypothalamus is suppressed by glucocorticoids, whereas in the placenta CRH production is stimulated by glucocorticoids. The human genome contains only one copy of the CRH gene, which has only one initiation site. We hypothesize that the observed differences in transcription regulation are due to tissue specific alterations in expression of transcription factors. Therefore, we sought to identify the key *cis*-acting elements and the transcription factors which regulate the human CRH gene.

We have used AtT20 cells as a model for inhibition of CRH expression by glucocorticoids in the hypothalamus. A human CRH promoter-reporter construct was developed and a series of deletions of the CRH promoter were used to identify *cis*-acting elements important for glucocorticoid and/or cAMP responsiveness. To identify transcription factors acting at these regulatory elements nuclear proteins were extracted and electrophoretic mobility shift assays (EMSA) performed. Mutational analyses were conducted to clarify the key *cis*-acting regulatory elements and the requirements for transcription factor binding.

In AtT20 cells two cAMP responsive regions were identified: (1) a consensus cAMP response element (CRE) and (2) a previously unrecognized caudal type homeobox response element (CDXRE). Overall cAMP-stimulated activity is due to the sum of the actions at these two sites. Glucocorticoids inhibited the component of the cAMP-stimulation occurring via the CRE (this action involves a negative glucocorticoid response element, nGRE, in the CRH promoter) but not that acting via the CDXRE. We also identified two regions of the CRH promoter that can be stimulated by glucocorticoids in AtT20 cells: (1) the CRE and (2) a region between -213 and -99bps. EMSA, using the human CRE, compared nuclear protein extracts from AtT20 with those from human primary placental cells (in which we have previously shown the CRE to be involved in stimulation of CRH by cAMP and by glucocorticoids). The transcription factors Jun and CREB were detected by EMSA-supershift in placental cells while in AtT20 extracts Fos and CREB were present. Furthermore, using a yeast one hybrid system a novel transcription factor with a leucine zipper motif was identified which bound to the CRE and is present in placental extracts (see abstract by KL Shipman et al).

Our results identify novel interactions between four *cis* regulatory sites in the CRH promoter important for regulation by cAMP and glucocorticoids, and indicate that the nuclear transcription factors binding to the CRE differ between the AtT20 cells and the placenta. We show that in the absence of specific interactions involving the nGRE the CRH promoter can be stimulated by glucocorticoids in AtT20 cells. The response of the CRH gene to factors acting via glucocorticoid receptors and cAMP is a reflection of the interactions of tissue specific transcription factors at the sites that have been identified.

## GALANIN-LIKE PEPTIDE (GALP) MRNA EXPRESSION IN POSTERIOR PITUITARY AND TESTIS OF THE RAT: REGULATION IN PITUITARY CELLS BY LACTATION

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Galanin-like peptide (GALP), originally identified in porcine hypothalamus, is a 60 amino acid peptide that shares sequence homology with galanin and has higher affinity for the GalR2 receptor than the GalR1 receptor (1). GALP mRNA expression is restricted to the arcuate nucleus and median eminence in normal rat brain (2, 3), whereas recent studies have revealed that GALP mRNA is expressed by pituitary cells in the neural lobe of the rat pituitary gland (4). Furthermore, pituitary GALP mRNA levels were significantly increased following osmotic stimulation (salt-loading and dehydration) (4). However, the expression of GALP in this tissue under other physiological conditions and in peripheral tissues such as testis, has not been investigated. Therefore, the current study examined the regional and cellular distribution of GALP mRNA in pituitary gland during lactation, and in the testis of adult rats, using *in situ* hybridization. Pituitary glands were removed from female rats that had been lactating for 7-14 days ( $\geq 10$  pups) and from age-matched, random-cycle, non-pregnant rats. Testes were collected from adult male rats. Cryostat sections were prepared and hybridized with specific [<sup>35</sup>S]-oligonucleotide probes for GALP and other relevant mRNA species. Slides were exposed to X-ray film and semi-quantitative densitometric analysis of pituitary sections was performed. Lactation produced a time-dependent increase in GALP mRNA in the neural lobe, with levels elevated by 11-fold after 7 days, and 10- and 7-fold increases at 11 and 14 days, respectively (ANOVA,  $p \leq 0.05$ ). Levels of vasopressin (AVP) mRNA in the neural lobe were also increased by lactation, consistent with earlier reports (5). Further studies are required to determine the presence of GALP immunoreactivity and GALP receptors in this region and possible actions on neurohormone release. In the testis, GALP mRNA-containing cells were identified in a *small* sub-population of seminiferous tubules in both film- and emulsion- autoradiographs. In contrast, AVP and oxytocin mRNAs, which are reportedly present in interstitial Leydig cells (6), were more widely distributed. Studies are in progress to determine the *identity* of the GALP-expressing cells within the testis and what factors influence its expression. Overall, the unique distribution of GALP-positive cells in mediobasal hypothalamus, pituitary neural lobe and testis suggests that this newly-discovered peptide may play an important role in the neuroendocrine regulation of various physiological functions, including osmotic homeostasis, lactation and reproduction.

## EFFECTS OF CYTOKINES ON HUMAN AND RAT MYOMETRIAL CONNEXIN 43 EXPRESSION DURING PREGNANCY

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Infection induced-preterm labour is associated with a rise in pro-inflammatory cytokines. However, the effects of cytokines on human myometrial function are presently unknown. The aim of this study was to identify effect of tumour necrosis factor alpha (TNF- $\alpha$ ) and other inflammatory cytokines on connexin 43 (Cx 43) expression in the human myometrium and also examine the *in vivo* effect of endotoxin on Cx 43 expression using the rat as a model. Gap junction formation by the Cx 43 protein is essential for synchronous myometrial contraction in human pregnancy. Recent evidence suggests that inflammatory cytokines and endotoxin affect connexin 43 expression in other tissues such as the heart, kidney and lungs. We have hypothesised that TNF- $\alpha$  and other inflammatory cytokines alter myometrial Cx 43 formation. Human myometrial biopsies were collected from women undergoing elective Caesarean section at term. The tissue was incubated with TNF- $\alpha$  and a number of different cytokines including the interleukins 1, 4, 5, 8, 13, TNF- $\alpha$  and GM-CSF. Connexin protein concentrations were measured using immunoblotting techniques. TNF receptor localisation in the myometrium was determined by immunohistochemistry. Both subtypes of TNF receptors are present in human myometrium at term, suggesting a possible paracrine or autocrine role of TNF- $\alpha$  in the uterus. TNF- $\alpha$  did not alter the expression of Cx 43 protein in the myometrial explants. However, Cx 43 protein levels were increased in the myometrium when incubated with interleukins 1, 5, 8, and 13. Furthermore, endotoxin administration to pregnant rats increased the expression of Cx 43 in the myometrium. These results suggest that systemic or locally produced cytokines may upregulate Cx 43 expression in the myometrium and contribute to the initiation of preterm labour in pregnancies associated with infection.

## EXPRESSION OF NEUROPEPTIDE Y & NEUROPEPTIDE Y RECEPTORS 1 & 5 (Y1 & Y5) IN THE HYPOTHALAMUS OF HIGH FAT-FED RATS

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Neuropeptide Y (NPY) is a potent stimulator of feeding but it is unclear which of its receptor subtypes, Y1, Y2, Y4 or Y5, mediates this effect. There is pharmacological evidence suggesting that both Y1 and Y5 may contribute to NPY's central effect on appetite and feeding behaviour. The aim of the current study was to determine if the hypothalamic expression of NPY, Y1 and Y5 was altered in a rodent model of high fat feeding. Male Wistar rats were fed *ad libitum* either a high fat diet (approx. 60% fat) or standard chow diet (12% fat) for 3 weeks. Under thiopental anaesthesia animals were perfused with paraformaldehyde, brains dissected and 5 $\mu$ m serial paraffin sections of the hypothalamus collected. Digoxigenin-labelled NPY, Y1 and Y5 antisense and sense riboprobes were generated (cDNAs kind gift from H. Herzog, Garvan Institute) and *in situ* hybridisation (ISH) performed using an alkaline phosphatase-conjugated, anti-digoxigenin antibody for detection. ISH was evaluated by quantifying the positively hybridising cells in defined nuclear regions. Immunohistochemistry was performed using an antibody to leptin receptor. Serum leptin levels were increased in fat-fed rats as compared to chow-fed controls. Leptin receptor immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN) was reduced in fat-fed rats compared to controls. Expression of NPY mRNA in the arcuate nucleus was similar in high-fat fed rats and controls, but Y1 and Y5 mRNA expression levels were significantly reduced. There was no change in Y1 or Y5 mRNA expression in the PVN or ventromedial nucleus with high fat-feeding, sites known to be involved in NPY's role in feeding. These results would be consistent with either, NPY not mediating the increased adiposity seen in association with a high-fat diet, or a failure of appropriate suppression of NPY in the face of increased serum leptin levels under these conditions. This could potentially be mediated via reduced leptin signalling resulting from reduced leptin receptor expression in the PVN. The reduction in Y1 and Y5 mRNA expression in the arcuate nucleus would be consistent with a homeostatic adaptation to reduce NPY signalling, and hence prevent excess weight gain, with a high fat diet.

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## INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS IN FOLLICULAR FLUID FROM HEALTHY AND ATRETIC BOVINE FOLLICLES

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Once follicular growth is initiated, bovine follicles either continue to grow and ovulate or they become atretic. Not all follicles grow at similar rates, leading to the concept of dominant and subordinate follicles. Bovine follicles less than 5 mm diameter have basal granulosa cells which are either rounded or columnar in shape. These follicles have either an aligned conventional follicular basal lamina or one of many loops. On atresia of follicles less than 5 mm, granulosa cells die first either near the antrum and spreading towards the basal lamina (antral atresia) or the converse (basal atresia). Insulin-like growth factor 1 and its binding proteins (BP 1 to 6) are important in follicular development and atresia. However, their expression has not been examined in the context of different phenotypes of healthy or atretic follicles, which was the aim of this study.

Follicles (n = 145; 2-5 mm) were collected from visually non-pregnant animals at an abattoir, dissected from the ovary, blotted to remove excess fluid, and then snap frozen. The frozen follicles were bisected and one half placed into 2.5% glutaraldehyde in MOPS buffer, and subsequently embedded in epoxy resin. Sections were cut and the follicles classified as healthy with rounded or columnar cells, or antral or basal atretic (n = 8-10 per group). The remaining parts of the frozen ovaries were thawed, and follicles were centrifuged (10,000g, 10 min, 4C) and the supernatants collected. Three microlitres of each follicular fluid was subjected to electrophoresis and BPs detected (based upon molecular weights) by ligand blotting (<sup>125</sup>I-labelled IGF II). Since a number of gels were required for the complete analysis, samples from each group were equally allocated to each gel, and an internal control was also included.

BP4 and BP5 levels were substantially and significantly ( $P < 0.001$ ) greater in atretic follicles. BP2 levels were slightly greater, and there was no significant difference in the levels of BP3 between healthy and atretic follicles. There were no significant differences between the two types of atresia for any BPs. In healthy follicles BP5 was significantly ( $P < 0.05$ ) lower in follicles with rounded basal cells than columnar cells. No other BPs were significantly different. The current results confirm previous studies on atretic versus healthy follicles, but also find differences between different phenotypes of healthy follicles.

## DISSECTING THE APOPTOTIC PATHWAYS OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN (IGFBP)-3 AND IGFBP-5 IN HUMAN BREAST CANCER CELLS.

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IGFBP-3 and IGFBP-5 modulate the mitogenic and anti-apoptotic effects of IGFs by regulating their interactions with the type I IGF receptor. However, both IGFBPs have been shown to have IGF-independent effects on cell growth and apoptosis. We have previously demonstrated the growth inhibitory and proapoptotic effects of IGFBP-3 in human breast cancer cells. Furthermore, IGFBP-3 sensitises these cells to radiation-induced apoptosis in a p53-independent manner<sup>1</sup>. Recent studies have emphasised the important role of the mitochondria in the regulation of apoptosis. Diverse apoptotic signals lead to the release of cytochrome c from mitochondria into the cytosol, where it promotes the activation of caspases – the molecular effectors of the apoptotic process. Members of the Bcl-2 family have been shown to influence the release of cytochrome c from mitochondria. In previous studies, we have shown that stable transfection of IGFBP-3 results in a significant increase in expression of proapoptotic Bax and Bad proteins, and a decrease in anti-apoptotic Bcl-2 and Bcl-x<sub>L</sub> proteins compared to vector controls<sup>1</sup>. Our current studies have aimed to further elucidate the apoptotic pathway initiated by IGFBP-3 and examine whether IGFBP-5 has similar or opposing effects. To examine the downstream apoptotic pathway induced by IGFBP-3, we have used an adenoviral-mediated transient transfection system. IGFBP-3 resulted in significant growth inhibition ( $p < 0.05$ ) and apoptosis (assessed by analysis of DNA fragmentation,  $p < 0.05$ ) in T47D and MDA-MB-231 human breast cancer cells. We are using this system to examine the effects of IGFBP-3 on mitochondrial-mediated apoptosis, namely the release of cytochrome c into the cytoplasm and activation of caspases-9 and -3. Changes in the intracellular localisation of Bcl-2 proteins are also associated with the induction of apoptosis, with increasing evidence of a role for Bax in the nuclear compartment as well as its more defined role in the mitochondria. IGFBP-3 expression resulted in a significant accumulation of Bax in the nucleus (up to 1.75 fold;  $p < 0.03$ ) compared to vector controls, determined by immunocytochemical staining. These results have been confirmed by cell fractionation studies and Western blotting. These studies are being continued to determine possible direct interactions between IGFBP-3 and members of the Bcl-2 family. In contrast to IGFBP-3, IGFBP-5 is reported to play an anti-apoptotic role in cancer cell growth. We have observed a significant survival effect in MDA-MB-231 breast cancer cells stably overexpressing IGFBP-5 ( $p < 0.02$ ) but, paradoxically, preliminary data suggest an enhanced apoptosis in the same cells when exposed to ionising radiation, accompanied by enhanced G2/M arrest and the formation of giant cells. The latter is associated with the induction of apoptosis and reported to occur by disruption of cell cycle checkpoints normally regulated by p21<sup>WAF1/CIP1</sup>. To further elucidate the effects of IGFBP-5 on the apoptotic process, we are examining the expression of p21 and the G2/M specific cyclin-dependent kinase p34/cdc2 in wild-type and IGFBP-5-overexpressing cells, both basally and following radiation. These effects of IGFBP-5 on the apoptotic process will be further delineated using the adenoviral-mediated transient expression system. Supported by NHMRC.

## INTER-DEPENDENCE BETWEEN TRANSFORMING GROWTH FACTOR-BETA (TGF- $\beta$ ) AND INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 (IGFBP-3) IN THEIR GROWTH INHIBITORY EFFECTS ON BREAST CANCER CELLS

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TGF- $\beta$  regulates the proliferation of its target cells by interacting with specific cell surface receptors, of which type I and II receptors are involved in TGF- $\beta$  signal transduction. Type I TGF- $\beta$  receptor (TGF- $\beta$ RI) is phosphorylated by type II TGF- $\beta$  receptor (TGF- $\beta$ RII) once it is bound to TGF- $\beta$ . Activated TGF- $\beta$ RI then phosphorylates and activates the receptor-regulated Smads, Smad2 and Smad3, which are the intracellular transducers of TGF- $\beta$  signaling. We have previously shown that in T47D human breast cancer cells transfected with TGF- $\beta$ RII cDNA (T47D/TGF- $\beta$ RII), IGFBP-3 stimulates Smad2 and Smad3 phosphorylation, and potentiates the phosphorylation of these intermediates by TGF- $\beta$ . Moreover, the study has shown an inter-dependence between exogenous IGFBP-3 and TGF- $\beta$  in their growth-inhibitory effect on breast cancer cells (1).

In this study, the ability of both TGF- $\beta$  and IGFBP-3 to activate the TGF- $\beta$  signaling pathway was further examined in T47D/TGF- $\beta$ RII cells. MCF-7 human breast cancer cells were used as a TGF- $\beta$ -responsive cell model since they possess intact TGF- $\beta$  signaling machinery. In T47D/TGF- $\beta$ RII cells, total TGF- $\beta$ RI levels were surprisingly down-regulated as determined by immunoblot of cell extracts. Nevertheless, exogenous TGF- $\beta$  (2.5 ng/ml) induced TGF- $\beta$ RI phosphorylation in a time-dependent manner in these cells, peaking at 5 mins, compared to a peak at 10 mins in MCF-7 cells. IGFBP-3 (500 ng/ml) also induced time-dependent phosphorylation of TGF- $\beta$ RI in both cell lines, peaking at 15 mins after exposure to IGFBP-3. Co-treatment with TGF- $\beta$  and IGFBP-3 resulted in a greater level of TGF- $\beta$ RI phosphorylation than caused by either agent alone. The observation that both TGF- $\beta$  and IGFBP-3 can activate TGF- $\beta$ RI explains the previously observed synergy between TGF- $\beta$  and IGFBP-3 in stimulating Smad2 and Smad3 phosphorylation. To examine whether this synergy extends to the activation of TGF- $\beta$ -dependent gene transcription, we used a reporter construct consisting of a TGF- $\beta$ -responsive segment of the human plasminogen activator-inhibitor (PAI)-1 promoter cloned upstream of the firefly luciferase reporter gene (PAI-1/L). TGF- $\beta$ -induced binding of Smads to Smad-binding elements in the PAI-1 promoter has been shown to induce PAI-1 activation. Both TGF- $\beta$  and IGFBP-3 were capable of inducing the luciferase activity, which indicates that IGFBP-3, similar to TGF- $\beta$ , is capable of inducing PAI-1 transcriptional activation.

These results indicate that, similar to TGF- $\beta$ , IGFBP-3 is also capable of inducing the TGF- $\beta$  signaling pathway, which requires the presence of an active TGF- $\beta$  signaling machinery. Whether IGFBP-3 acts independently or through a direct interaction with TGF- $\beta$ RI or TGF- $\beta$ RII is not clear.

## STRUCTURAL AND FUNCTIONAL CHARACTERISATION OF THE INTERACTION OF INSULIN-LIKE GROWTH FACTORS WITH VITRONECTIN - A NOVEL IGF-REGULATORY PROTEIN

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We have conducted structural and functional studies to further investigate our recent discovery that insulin-like growth factor-II (IGF-II) binds to vitronectin (VN) (1), an intriguing multi-functional protein that interacts with integrins and is pivotal to a number of fundamental cellular processes including cell attachment, migration and cell survival. Structural studies examining binding of labelled IGF-II to VN have revealed that positively-charged amino acid residues in the C-domain of IGF-II mediate the direct binding of IGF-II to VN; amino acids in IGF-II important for binding to the type-I IGF receptor are not involved; plasminogen activator-inhibitor-1 competes for binding of IGF-II to VN; and IGF-II appears to bind to "extended" and not to "native" VN. This last result is especially interesting as VN is predominantly in the polymerised "extended" conformation in the extracellular context. Unlike IGF-II, IGF-I does not bind to VN. However, review of the literature revealed that binding of VN to integrins is essential for a range of IGF-I-stimulated biological effects including DNA synthesis, IGF binding protein-5 (IGFBP-5) production, type-I receptor autophosphorylation and cell migration (2). This prompted us to hypothesise that an interaction between IGF-I and VN may be mediated through the IGFBPs. Structural studies examining binding of radiolabelled IGF-I to VN in the presence and absence of IGFBPs revealed interesting results. IGFBP-5 was shown to significantly ( $p < 0.05$ ) enhance binding of IGF-I to VN while IGFBP-2 had minimal effects. Furthermore, radiolabelled-IGF-I binds to VN in the presence of non-glycosylated IGFBP-3, yet only minimal binding is observed if glycosylated IGFBP-3 is used. Using the same binding assay protocol with radiolabelled IGF-II revealed that IGF-II could also bind to VN via IGFBPs, in addition to binding to VN directly. To further examine the interactions of IGFs with VN, we translated these structural findings to functional studies. Assays examining the incorporation of [<sup>3</sup>H]-leucine into newly synthesised protein in HaCAT human keratinocytes in response to IGFs demonstrated significant synergistic responses ( $p < 0.05$ ) when IGF-II was pre-bound to VN. Synergistic responses, albeit small, were also found with the cells when IGF-I was pre-bound to VN in the presence of IGFBP-5. Taken together, these studies suggest that IGFBPs are important factors mediating interactions between IGFs and VN in the extracellular environment. Further analyses in these cells are being conducted to determine if other aspects of cell function such as cell attachment, migration and survival are also modified by the IGF/IGFBP/VN interaction.

Upton Z *et al.* (1999) *Endocrinology* 140: 2928-2931

Clemmons DR *et al.* (1999) *Endocrinology* 140: 4616-4621

## SEX DIFFERENCES IN THE SKIN MICROVASCULAR RESPONSE TO CORTICOTROPIN-RELEASING HORMONE

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Life expectancy is greater in women than in men, predominantly due to the lower incidence of cardiovascular disease. Clinically evident vascular disease rarely occurs in premenopausal women, but after menopause its incidence increases dramatically. A number of hormones are differentially expressed between males and females and this may contribute to the differences in vascular function.

Corticotropin-releasing hormone (CRH) is a potent vasodilator in a number of vascular beds and plays a central role in orchestrating the hypothalamic-pituitary-adrenal (HPA) axis and stress response. However skin also expresses CRH-like peptides and CRH receptors, suggesting a potential role for the peptide in vasoregulation of the dermis.

As stress is a major contributor to the development of cardiovascular disease and CRH is differentially expressed between the sexes, we hypothesised that CRH may have sex-specific vascular effects in the microvascular circulation. To test this hypothesis we determined whether the vasoactive effects of CRH are influenced by sex (males n=5, females n=14), and in females, the different stages of the menstrual cycle; early follicular (n= 4), mid-cycle (n=6), or luteal phase (n=4). CRH (1nM) was administered subcutaneously by iontophoresis to a small area of skin in the forearm and laser Doppler flowmetry measured blood flow in the same area simultaneously.

CRH relaxed the microvascular circulation in both males and females in a dose-dependent fashion. However, the dilatory response to CRH was more potent in the females (44.15 ±18.5 perfusion units (PU)) when compared to the males (13.13 ±1.97 PU, p<0.05). Furthermore, this effect varied during the menstrual cycle with a more potent relaxation observed at midcycle (81.15 ±39.3 PU) than early follicular (8.63 ±0.99 PU) or luteal phase (24.17 ±10.34 PU). CRH-induced dilation was blocked by the CRH antagonist,  $\alpha$ -helical CRH<sub>9-41}</sub> (10nM), suggesting that CRH acts via its receptor to increase microvascular dilation. The data are consistent with an action that is modulated by estrogen.

As a control study, acetylcholine (1nM) a well characterised dilator agent was administered to males (n=6) and females (n=6). No sex difference in microvascular dilation was observed (males 163.3 ±20.8PU and females 181.38 ±21.1PU). These studies demonstrate for the first time that CRH is a sex-specific vasodilator and that these effects vary during the menstrual cycle.

## INHERITED CORTICOSTEROID BINDING GLOBULIN DEFICIENCY IS ASSOCIATED WITH INCREASED PROLIFERATION AND ENHANCED DIFFERENTIATION OF HUMAN PREADIPOCYTES *IN VITRO*.

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Corticosteroid binding globulin (CBG) is a cortisol carrier protein produced by the liver and in other tissues including human kidney and placenta. We recently discovered a family with the first described complete loss-of-function (null) CBG mutation and have noted that family members who are homozygous for the null mutation (CBG<sup>-/-</sup>) were relatively obese compared with other family members. There is preliminary evidence for a cellular role for CBG, possibly modulating glucocorticoid action. We have found that normal human adipocytes and preadipocytes (PAs) express CBG. As PA growth and differentiation is glucocorticoid dependent we compared PAs obtained by abdominal subcutaneous biopsy from a 57 yr old CBG<sup>-/-</sup> male (BMI 39.4, waist 125cm) with normal (CBG<sup>+/+</sup>) subcutaneous male PAs. We compared replication, basal and peroxisome proliferator activated receptor  $\gamma$  (PPAR $\gamma$ ) activator-mediated differentiation, glucocorticoid receptor characteristics and type 1 11 $\beta$ -hydroxysteroid dehydrogenase activity. CBG<sup>-/-</sup>PAs were morphologically indistinguishable from CBG<sup>+/+</sup>PAs. CBG<sup>-/-</sup>PAs proliferated more quickly, as assessed by change in 490nm absorbance over 24hrs by formazan colorimetric assay. CBG<sup>-/-</sup>PAs absorbance change was 0.193±.013 (n=8), compared with 0.066±0.003 (n=8, P < 0.0001) for CBG<sup>+/+</sup>PAs that had been grown *in vitro* for the same period of time, or 0.074±0.014 (n=8, P < 0.0001) for CBG<sup>+/+</sup> PAs that had been passaged the same number of times. The addition of cortisol or RU486 (10-500nM for 72 hours) did not influence the rate of replication of CBG<sup>-/-</sup> or CBG<sup>+/+</sup> PAs. The increased replication rate was not associated with more rapid senescence. CBG<sup>-/-</sup> PAs differentiated well in serum-free medium containing cortisol, insulin and rosiglitazone (a PPAR $\gamma$  activator), as assessed by the accumulation of lipid and glycerol-3-phosphate dehydrogenase(G3PD) activity. The observed lipid accumulation was subjectively greater than for CBG<sup>+/+</sup>PAs, and the G3PD activity for CGB<sup>-/-</sup> PAs was 1334 milli-units/mg protein compared with 68, 118 and 254mu/mg for 3 CBG<sup>+/+</sup>PA samples. There was no difference in basal differentiation in serum free medium with cortisol and insulin without rosiglitazone, G3PD activity CBG<sup>-/-</sup> PAs 47mu/mg, compared with CBG<sup>+/+</sup> PAs 14, 36 and 68 mu/mg. Glucocorticoid receptor characteristics were normal by dexamethasone whole cell binding, 3 assays on 2 strains, Kd= 6.8, 9.5 and 12.1nM and Bmax= 263, 488 and 603 fmol/mg compared with CBG<sup>+/+</sup>PAs Kd= 5.1-12.6nM and Bmax= 327-599fmol/mg (n=6). 11 $\beta$ -hydroxysteroid dehydrogenase activity was normal by HPLC of cortisol/cortisone levels after a 6 hour incubation of PA monolayers with 500nM cortisol or cortisone. Cortisone to cortisol conversion for 2 CGB<sup>-/-</sup> PA strains was 14 and 22 fmol/mg protein/hr, compared with CBG<sup>+/+</sup>PAs 10, 10 and 18 fmol/mg/hr with minimal cortisol to cortisone activity for both.

These findings suggest that CBG has a role in regulating PA replication and PPAR $\gamma$ -mediated differentiation but not basal differentiation, at a cellular level. The mechanism of these effects is not established with evidence against differences in glucocorticoid receptor complement or intracellular cortisol metabolism but an interaction between CBG and PPAR $\gamma$  is suggested.

## PROTEIN METABOLISM IN CUSHING'S DISEASE

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Glucocorticoids (GC) exert an acute catabolic effect by increasing proteolysis and increasing irreversible protein loss through oxidation. Whether these perturbations in protein metabolism are sustained in chronic GC excess is unknown. To address this question, we have studied whole body protein turnover in 10 patients with active Cushing's disease and 11 normal subjects. Whole body protein turnover was measured using a 3-h primed constant infusion of 1-[13C] leucine, from which rates of leucine appearance (Ra, an index of protein breakdown), leucine oxidation (Lox, an index of irreversible oxidative loss of protein) and non-oxidative leucine disposal (NOLD, an index of protein synthesis) were estimated. Lean body mass (LBM) and fat mass (FM) were measured by dual energy x-ray absorptiometry.

	Body weight (Kg)	%LBM	% body fat	Ra (umol/min/KgFF M)	Lox (umol/min/KgFF M)	NOLD (umol/min/Kg FFM)
Cushing's	74.0 ± 5.1	57 ± 2*	43 ± 2*	3.09 ± 0.14*	0.61 ± 0.08	2.48 ± 0.13*
Controls	68.0 ± 3.6	69 ± 3	31 ± 3	2.67 ± 0.09	0.59 ± 0.03	2.08 ± 0.07

Percent LBM was significantly lower, and percent FM significantly higher in subjects with Cushing's disease. In both normal subjects, and subjects with Cushing's disease, LBM was a major, independent determinant of leucine Ra ( $r = 0.79$  Cushing's,  $r = 0.86$  Controls,  $p < 0.002$ ), Lox ( $r = 0.83$  Cushing's,  $r = 0.81$  Controls,  $p < 0.002$ ), and NOLD ( $r = 0.65$  Cushing's,  $r = 0.82$  Controls,  $p < 0.05$ ). After correcting for LBM, Cushing's patients had significantly higher rates of leucine Ra, and NOLD, while Lox did not differ between the two groups.

In summary, when corrected for lean body mass, proteolysis and protein synthesis are increased in subjects with Cushing's disease while protein oxidation is unchanged. We conclude that (i) LBM is a major determinant of whole body protein metabolism in normal subjects and subjects with Cushing's disease, (ii) perturbations of chronic GC excess are different from those of acute GC excess, (iii) normalisation of irreversible protein loss through oxidation acts to conserve lean body mass in Cushing's disease. The mechanism resulting in this metabolic adaptation is not known. (Supported by NHMRC of Australia)

## EVIDENCE AND POSSIBLE ROLE OF GROWTH HORMONE SECRETAGOGUES RECEPTOR AND GHRELIN IN HUMAN ENDOMETRIAL TISSUE REMODELLING PROCESS

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Since the discovery that met-enkephalin stimulates GH release from the anterior pituitary in 1980s, small synthetic peptide and non-peptide molecules, called GH secretagogues (GHSs), have been developed. GHS receptor (GHS-R), a G-protein coupled seven-transmembrane-domain receptor, is present in the pituitary and hypothalamus. Endogenous ligand for GHS-R, named Ghrelin, has recently been found in rat hypothalamus. Ghrelin has also been found in several peripheral tissues including the heart and the kidney. Human endometrium is an unusual adult tissue undergoing extensive tissue remodelling with each menstrual cycle. Such a remodelling process requires participation of many biologically active systems. The aim of this study is to establish whether the GHS-R and its ligand, ghrelin, are expressed and whether there are changes in the human endometrium across the menstrual cycle. Reverse transcriptase (RT) polymerase chain reaction (PCR) for the GHS-R and immunohistochemical staining using highly specific antisera for the GHS-R and Ghrelin was performed. The intensity of the immunostaining was scored semi-quantitatively from (-) negative to (++++) in relation to known positive and negative controls. RT-PCR was employed to detect GHS-R mRNA in human endometrial tissues from day 1, 8, 13 and 21 of the menstrual cycle. The highest level of GHS-R mRNA was observed in the sample from day 21 of the menstrual cycle. There were lower levels of expression observed in day 13 but no detectable expression on day 8 or day 1. GHS-R immunohistochemical staining was confined predominantly to epithelial and stromal cells in endometrial sections sampled from day 8, 13, 21 of the menstrual cycle. The greatest intensity of staining was observed in samples from day 21 of the menstrual cycle, while samples from day 13 showing very little staining and samples from day 8 did not stain at all. This was consistent with the RT-PCR data. Ghrelin immunoreactivity was observed in human endometrial sections sampled from day 21 of the menstrual cycle and was present in epithelial, stromal and perivascular cells. This is a broader cellular distribution than that determined for the receptor and suggests a paracrine role of ghrelin in the tissue. Based on the above results, we conclude that GHS system exists in human endometrium. Since the highest level of both the receptor and ligand are present on day 21, during the "window of receptivity" a functional involvement of this system in uterine receptivity could be proposed. (Supported by NHMRC (Grant number 143798 and 143802) and US Human Growth Foundation).



## MILK PROLACTIN AND THE DEGREE OF BREAST FULLNESS IN FULL-TERM BREASTFEEDING WOMEN

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It has previously been shown (Cox *et al.*, 1996, *Exp. Physiol.*, **81**:1007) that the concentration of prolactin in women is higher in fore- (F) than in hind (H)-milk (F-H gradient). It was hypothesised that this gradient was due to cyclic entry of prolactin into the milk, such that prolactin uptake into the alveoli was inhibited when the breast was full. Therefore the aim of this study was to determine the relationship between the concentration of prolactin in milk and the degree of breast fullness. Full-term breastfeeding women (n=15; 1 to 6 months post-partum) measured their 24h milk production by test-weighing their infant (Arthur *et al.*, 1987, *J. Ped. Gastroenterol. Nutr.*, **6**:758) and collected F and H milk samples at each feed. Subsequently these women attended our laboratory where one breast was expressed using a MEDELA electric breast pump for 5 minutes, collecting milk samples every 30 seconds. After being allowed to refill, the same breast was again expressed, and milk samples collected as before. The prolactin concentration of all skim-milk samples was determined via IRMA [<sup>125</sup>I] and the degree of breast fullness was calculated (Daly *et al.*, 1993, *Exp. Physiol.*, **78**:209).

**24h Study:** The mean ( $\pm$ SD) concentration of milk prolactin for all women (n=15) over the 24h period was  $18.5 \pm 11.6$   $\mu$ g/L (F) and  $16.8 \pm 12.8$   $\mu$ g/L (H). The variation between women masked small changes within women in the concentration of milk prolactin over the 24h period, therefore a prolactin ratio (individual F- or H-value divided by the mean for all F- or H-samples of the 24h period) was determined. The prolactin concentration was highest in the F- and H-milk between 02:01 to 06:00h (prolactin ratio for F and H, 1.5), with the lowest concentrations occurring between 10:01 and 18:00h (prolactin ratio for F and H, 0.8). Furthermore, we observed a relationship between the F-H gradient and both the time of day (was greatest between 06:01 and 10:00h; 4  $\mu$ g/L) and with time since the last feed (was greatest between 9.1 to 12.0h since the last feed; 6.5  $\mu$ g/L). In addition, we observed that when duration between feeds was the greatest (06:01 to 10:00h) the volume of milk removed was the highest. We concluded that when the breast is most drained of milk (in the late-evening), and the rate of milk synthesis is greatest, the F-H gradient in the milk of the following feed (in the early-morning) is highest. This is consistent with the observation that prolactin uptake from the blood is dependant on the fullness of the breast, such that prolactin uptake may be inhibited in full alveoli. **Expression Study:** Despite there being a significant change ( $p < 0.05$ ) in the degree of breast fullness during the course of the 5-minute expression episode, no significant change ( $p > 0.05$ ) in the concentration in milk prolactin was observed, presumably due to the 24h variation described above. However a significant difference ( $p < 0.05$ ) between primiparous and multiparous women was observed in the percent change in the concentration of milk prolactin over the expression episode (mean  $\pm$  SEM;  $29.4 \pm 7.9\%$ , and  $9.9 \pm 2.7\%$  respectively). Furthermore the primiparous women, demonstrated a correlation ( $p < 0.05$ ;  $n = 14$ ;  $r = -0.55$ ) between the F-H gradient and stage of lactation, whereby prior to 3 months post-partum the F-H gradient was positive and between 3 and 6 months post-partum it was negative. Despite this variation, milk production remains constant for the first 6 months of lactation suggesting that prolactin is permissive for, rather than a regulator of, milk synthesis. We therefore hypothesise that in early lactation primiparous women have a higher requirement for prolactin than do multiparous

## NEONATAL STRESS POTENTIATES HPA ACTIVITY AND ENHANCES TUMOR METASTASIS IN ADULT RODENTS.

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Exposure to neonatal stress can permanently alter the HPA-axis, resulting in potentiation of the HPA response to stress in adulthood. Given the interactions between glucocorticoids and the immune system we investigated the long-term impact of two neonatal interventions; maternal separation (MS) and endotoxin exposure (EE) on growth, stress responsivity, and resistance to tumor colonization in adulthood in F344 rats. In Exp.1, neonates were subjected to MS (2hr/day) for the first 21 days postnatal. Control animals were left undisturbed. In Exp 2. Neonate pups were injected with bacterial endotoxin (0.05mg/kg salmonella enteritidis, i.p.) or the vehicle (phosphate buffered saline) on days 3, 5 and 7 post-partum. At 90 days of age animals were inoculated with MADB106 tumor cells ( $1 \times 10^5$ ). Three days prior to and after tumor administration animals underwent a 10hr period of restraint. A control group of animals was unrestrained during the same time period. Blood was obtained after the restraint period for assessment of plasma corticosterone. Tumor colonization was assessed 3 wks after inoculation, at which point blood was also obtained for assessment of Natural Killer (NK) cell activity. Data analysis indicates that MS and EE produced significant ( $p < 0.05$ ) delays in weight gain that persisted into adulthood. Neonatal MS and EE was also associated significantly ( $p < 0.05$ ) higher stress-induced corticosterone levels in adulthood. Finally, neonatal MS and EE exposure was associated with a significant increase in tumor cell metastasis ( $p < 0.001$ ) and a significant impairment in the activity of NK cells ( $p < 0.01$ ), cells critically involved in the early surveillance and eradication of tumor cells. In summary, neonatal stressors impede growth and impair tumour immunity, and are associated with alterations in the HPA response to stress in adulthood. The findings of this study are significant in that they indicate that neonatal exposure to a bacterial stimulus, or maternal separation, both common occurrences for human neonates, may have long term implications for health, with specific reference to cancer susceptibility.

## CORRELATION BETWEEN SALIVARY, CAPILLARY AND PLASMA CORTISOL LEVELS IN NORMAL SUBJECTS AND HYPOADRENAL PATIENTS ON REPLACEMENT THERAPY

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**BACKGROUND:** In patients with hypoadrenalism, it is often difficult to assess the adequacy of glucocorticoid replacement therapy. Serial serum cortisol measurement is the most frequently test used. This measures the total but not free (bioactive) cortisol level. Salivary cortisol has been found to correlate closely with plasma free cortisol, which is the true indicator of glucocorticoid activity. It is independent of salivary flow, and involves a stress free collection method. Blood spot sample collected by finger-prick also offers a quick and simple way of assessing capillary blood cortisol levels. **AIM:** To assess the correlation between total serum cortisol, salivary cortisol and blood spot capillary cortisol obtained simultaneously from normal subjects as well as patients who are on hydrocortisone replacement for hypoadrenalism (primary and secondary). **METHODS:** 8 normal, relaxed subjects were recruited to provide saliva (by chewing on a salivette device), venous blood (by venipuncture) and blood spot (by finger-prick) simultaneously at one time point. 8 hypoadrenal patients on hydrocortisone replacement therapy (HAPR) were brought into hospital to have the same sampling done at 8 set time points of the day. These time points were chosen to reflect the cortisol levels prior to, 1-hour and 2-hours after the patients' hydrocortisone dose. Salivary cortisol and capillary cortisol were both measured by radioimmunoassay, but serum cortisol was measured using chemiluminescent techniques (Immulite). **RESULTS:** For the normal subjects, blood spot capillary cortisol correlated very strongly with total serum cortisol ( $R^2=0.99$ ), while the salivary free cortisol also had a fair correlation with serum cortisol ( $R^2=0.83$ ). However, for HAPR, while some association between capillary and serum cortisol existed, the relationship between salivary cortisol and plasma cortisol was less apparent. **DISCUSSION:** The strong correlation between blood and saliva concentrations of cortisol seen in normal individuals was not seen in patients on replacement treatment. Oral dosing probably causes a rapid saturation of CBG and marked fluctuations in equilibrium of bound:free cortisol. Saliva and blood spot sampling provide a convenient and simple way to measure cortisol levels at multiple times in a single day. We will discuss the utility of these methods in determining appropriate dosage of glucocorticoid replacement.

## RELAXIN DEFICIENT MICE HAVE UNDERDEVELOPED MALE REPRODUCTIVE TRACTS

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Relaxin (RLX) is a polypeptide hormone primarily produced by the corpus luteum (ovary) of pregnancy and is best known for its actions on collagen and extracellular matrix remodeling. To establish the physiological function of RLX, the RLX knockout mouse was recently established and shown to have deficiencies in mammary gland and nipple development<sup>1</sup>. In the present study, the phenotype of the RLX knockout mouse was examined for effects on normal growth and remodeling of target tissues, with particular attention to the male reproductive tract.

The body weights of RLX wildtype (+/+), heterozygous (+/-) and homozygous (-/-) mice were measured from 1 week to 3 months of age, before animals were sacrificed at 1 week, 1 month and 3 months of age for tissue collection and histological analysis. Serial sections from the male reproductive tract (including the testes, epididymis, prostate, seminal vesicle) were examined after staining with H&E, Masson's trichrome and immunostaining with antibodies to detect apoptosis (Bax, Caspase-9) and cell proliferation (PCNA).

RLX -/- mice were significantly smaller than their wildtype counterparts at 1 and 2 months of age ( $p<0.05$ ), at a time when the mice were undergoing rapid growth. By adulthood (3 months of age), the difference in body weights was insignificant ( $n=20$  males/females for each group). No significant differences in the immature male reproductive tract were noted at 1 week or 1 month of age, however, by 3 months of age, reproductive organs from RLX -/- male mice were significantly smaller ( $p<0.05$ ) than those of RLX +/+ males ( $n=10$  for each group). Each individual organ of the RLX -/- reproductive tract was found to be smaller in size at 3 months of age. Upon histological examination of the male reproductive tract of RLX -/- mice, we found decreased sperm maturation (testis), increased collagen (testis, epididymis, prostate) and decreased epithelial proliferation of the prostate, compared to samples obtained from RLX +/+ animals. In the testis of RLX -/- mice the level of sperm maturity in adult (3 month) RLX -/- mice resembled that of immature (1 month) RLX +/+ animals. The marked differences in the extracellular matrix of the testis and prostate in RLX -/- males also correlated with an increase in the rate of cell apoptosis and an age-dependent accumulation of fibrotic tissue within these organs. RLX expression was demonstrated in the prostate gland and was elevated in 14 month old mice, suggesting that it plays more of a prominent role within the male reproductive tract, as mice age.

These data demonstrate that RLX is an important factor in the development and function of the male reproductive tract of mice and has an essential role on the growth of the prostate. Based on these observations, we postulate that RLX not only plays a previously unexpected role in normal mammalian growth, but may enhance male fertility. Relaxin may mediate its effects on growth and development, in part, by serving as both an anti-apoptotic and anti-fibrotic factor.

## TESTICULAR GERM CELL DEVELOPMENT DURING TESTOSTERONE ± PROGESTIN MALE CONTRACEPTION†

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Testosterone (T)-based contraceptive regimens may be further improved by the addition of progestin to more profoundly suppress gonadotrophins and sperm counts. This study investigated the effects of T with or without progestin administration on the time course and extent of suppression on sperm counts, gonadotrophins and testicular germ cell populations in normal men. T enanthate (TE, 200mg im weekly) ± depot medroxyprogesterone acetate (DMPA, 300mg im once) was given to normal men for 2, 6 or 12 weeks (n=5/group) prior to vasectomy and testicular biopsy. A control group of untreated men (n=5) was also included. Germ cell numbers were determined using the optical disector stereological technique and expressed per Sertoli cell and as a percentage of the control group. Serum and semen samples were taken weekly and serum FSH/LH measured by ultrasensitive Delfia assay methods. The inclusion of DMPA led to a more rapid fall in LH and FSH compared to the TE alone group (LH ED<sub>90</sub>: 3.4 ± 1.7 vs 9.9 ± 3.3 days [p<0.0001]; FSH ED<sub>90</sub> 7.9 ± 1.4 vs 12.6 ± 2.6 days [p<0.0001] n=15) but there was no difference in the extent of gonadotrophin suppression, with a maximum suppression of LH and FSH in both groups to ~0.3% and ~1.4% of baseline, respectively. There was no difference in the rate of suppression of sperm counts (ED<sub>90</sub>: 25.3 ± 13.9 vs 23.7 ± 7.3 days, TE+DMPA vs TE alone, n=10/group, NS) nor the extent of suppression (4/5 vs 5/5 men achieving <0.5million/ejaculate after 11 weeks). After 2 weeks of treatment, type B spermatogonia and early spermatocytes were lower in the TE+DMPA group, which was attributed to lower LH and FSH levels, but there were no differences in other germ cells. After 6 weeks, germ cells from type B spermatogonia through to pachytene spermatocytes were significantly less than control in both TE+DMPA and TE alone groups, however round to elongated spermatid numbers were not yet affected. The fact that there were normal numbers of mature elongated spermatids in the testis in both groups at 6 weeks, yet sperm counts were <10% of baseline in the week prior to biopsy suggests near-complete failure of sperm release (spermiation) in the testes of all men in the first 5 weeks of treatment. After 12 weeks, all germ cells from Apale (Ap) spermatogonia through to elongated spermatids were significantly affected, with a major lesion in the transition of Ap to B spermatogonia. Elongated spermatid number remained at ~20% of control, yet sperm counts were maximally suppressed to ~1% of baseline in both groups suggesting that spermiation failure is a feature of chronic, as well as acute, gonadotrophin suppression. There were no differences in any germ cell types with the inclusion of DMPA at either the 6 or 12 week time points.

We conclude that the addition of DMPA to TE induces a more rapid fall in gonadotrophins, and thus causes a more rapid decrease in B spermatogonia numbers, however the rate of onset and extent of germ cell and sperm count suppression is not altered. The data shows that impairments in spermatogonial development and sperm release are the major lesions to spermatogenesis in either TE+DMPA or TE alone contraception.

## CONVERSION OF TESTOSTERONE TO ESTRADIOL IS NOT THE MECHANISM BY WHICH TESTOSTERONE SUPPRESSES SPERMATOGONIAL NUMBER IN THE GnRH-IMMUNIZED RAT

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High levels of exogenously administered testosterone (T) inhibit the recovery of type A/intermediate spermatogonial (Sg) number in the gonadotrophin deplete rat (1). Similarly high testicular T levels have been associated with a detrimental effect on Sg development in the irradiated rat (2). Given that estradiol is a metabolite of T and that estrogen receptor β have been localised to Sg, we hypothesize that the mechanism of T-induced Sg suppression is mediated by estrogen action. Therefore this study aimed to determine the effects of co-administration of high dose T with an estrogen receptor antagonist on the restoration of early germ cell number in the GnRH-immunized rat. GnRH-immunised adult Sprague Dawley rats (n=7-8/group) received T, either in the form of a Silastic implant (24cm) or an injectable ester (Sustanon 100, 25mg sc every 3<sup>rd</sup> day) for 10 days alone or in combination with the estrogen receptor antagonist ICI 182,780 (2mg/kg in peanut oil, sc daily, Tocris, England). Control rats received peanut oil. Testes were perfusion-fixed and germ cells were quantified by the optical disector technique.

GnRH-immunization reduced type A/intermediate Sg and type B Sg/preleptotene spermatocyte (Sc) number (56% of control, P<0.05), while leptotene/zygotene Sc number was reduced to 63% (P<0.05) of control. As expected, T treatment did not significantly increase type A/intermediate Sg number (67% of control) compared to GnRH-immunized controls. Similarly, no difference was observed in the number of type A/intermediate Sg in rats treated with T in the presence of the estrogen receptor antagonist. Type B Sg/preleptotene Sc and leptotene/zygotene Sc numbers were increased (~83% of control, P<0.05) in T-treated rats compared to GnRH-immunized controls, although significance was not achieved for the number of type B Sg/preleptotene Sc in T24cm-treated rats (~73% of control). The T-induced increase in Type B Sg/preleptotene Sc and leptotene/zygotene Sc numbers were not effected by the addition of the estrogen receptor antagonist. This data demonstrates that inhibition of oestrogen action did not affect the restoration of type A/intermediate Sg number in the T-induced GnRH-immunized rat suggesting that the conversion of T to estradiol is not the mechanism by which high dose T inhibits Sg development. Exogenous T increased type B Sg and primary Sc numbers suggesting that T provides some support to these cell types, possibly by promoting cell survival in the short term.

## ACUTE GONADOTROPHIN ADMINISTRATION RESPONSE OF SERUM INHIBIN B AND PRO- $\alpha$ C IN NORMAL ADULT MEN

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The proposed reciprocal relationship between inhibin B (inh B) and follicle stimulating hormone (FSH) has been difficult to establish. It is known that inhibin exists in two biologically active forms, inhibin A and B, and that these isoforms share a common  $\alpha$  subunit linked to either a  $\beta$ A or  $\beta$ B subunit by a disulfide bond. It is also recognised that there are both larger precursor forms and smaller more processed forms of inhibin that exist in biological fluids thereby increasing the potential complexity of inhibin physiology. (1) Inh B and related proteins are secreted by both Sertoli and Leydig cells under the influence of gonadotrophins although the relative roles of FSH and luteinizing hormone (LH) and the cellular sources of these inhibin forms are unclear. Stimulation of inh B (105%) and pro- $\alpha$ C (60%) has previously been reported with a single dose of recombinant human (rh) FSH. Serum FSH levels peaked 12 hours after the injection of rhFSH while inhibin levels began rising at 24 hours and reached a peak within 72 hours. (2) However, there has been no published data on the dose response effect of acute FSH administration on inhibin secretion.

This study aimed to define the response of inhibin forms to increasing doses of (rh) FSH and the effect of co-administration of LH (in the form of hCG). Twenty-five normal men, 21-45 yrs were randomly assigned to one of 5 treatments (n=5/group): a single sc injection of 600, 1200 or 2400 IU rhFSH, 5000 IU hCG or 1200 rhFSH plus 5000 hCG. Blood samples were drawn prior to injection (Day 0) and then daily thereafter for 7 days. Serum was analysed for testosterone, FSH, LH, inh B and pro- $\alpha$ C. 600IU rhFSH did not significantly effect inh B or pro- $\alpha$ C levels. Peak inh B levels were reached by days 4-5 with 1200IU rhFSH (30% above baseline) and by days 2-7 with 2400IU rhFSH (38-54% above baseline). Peak pro- $\alpha$ C levels (40-85% above baseline) were reached with both 1200 and 2400 IU rhFSH by days 2-7. Inh B was not significantly increased by hCG. However pro- $\alpha$ C levels (45% above baseline) rapidly rose by day 1 with the administration of 5000IU hCG. 1200IU rhFSH plus 5000IU hCG led to an additive increase in pro- $\alpha$ C levels (95-98% above baseline) on days 4-5.

We conclude that in normal men high doses of FSH (1200-2400IU) stimulated a smaller rise in inh B and a similar rise in Pro- $\alpha$ C to previously reported.(2) We presume that both these FSH-inducible inhibin responses are of Sertoli cell origin. hCG acts only on Pro- $\alpha$ C consistent with a Leydig cell action and with a more rapid time course of response than that seen with the FSH-Sertoli cell axis. The apparent additive effects of hCG and FSH suggest dual and independent actions on Leydig and Sertoli cell components, respectively.

## A MOUSE MODEL OF SPINAL BULBAR MUSCULAR ATROPHY (SBMA)

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SBMA is a late onset, progressive neuromuscular disorder, characterised by wasting of the proximal muscles and signs of androgen insensitivity, including testicular atrophy and infertility. The underlying genetic mutation is the expansion of a trinucleotide repeat (CAG repeat) in exon 1 of the androgen receptor (AR) gene. This disorder is one of a group of neurodegenerative diseases associated with expanded CAG repeats, which includes Huntington Disease (HD) and several spinocerebellar ataxias. The CAG repeat in the AR is polymorphic, ranging from 8-31 repeats in normal individuals, with the disease phenotype associated with more than 38 CAG repeats. An experimental animal model of SBMA has been generated carrying a full-length human AR cDNA with 20, 65, or 120 CAG repeats. These cDNAs are driven by the cytomegalovirus promoter, resulting in expression in most tissues, with the notable exception of germ cells. Mice with either 65 or 120 CAG repeats displayed progressive behavioural and motor dysfunction. This was more dramatic in the mice with the longer repeats. Specifically, mice carrying 65 CAG repeats (10 lines) displayed a hind-foot clenching behaviour similar to that reported for the mouse models of HD. The mouse lines carrying 120 CAG repeats (3 lines) also displayed this foot clenching behaviour, which progresses to involve the forelimbs and eventually a full body clench. These mice also exhibited a marked reduction in cage activity, reminiscent of the behavioural abnormalities exhibited by HD mice. By 4 months of age, signs of a progressive muscle weakness are apparent. Muscle wasting, both unilateral and bilateral, was observed in these mice. Stereological analysis of the number of motor neurons in the lumbar enlargement showed a progressive loss in the mice carrying 120 CAG repeats when compared to wild type or mice with 20 CAG repeats. Neither wild type mice nor those with 20 CAG repeats showed any of the behavioural or motor dysfunction.

## IN VITRO PROPAGATION OF SERTOLI CELLS FOR TRANSPLANTATION

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The use of cell-based therapies to treat disorders such as Parkinson disease, Huntington disease, stroke and diabetes is severely hampered by the problems of graft rejection. Drug-based immunosuppressive therapies are an unattractive method for overcoming these problems. In the past five years, several groups have identified the Sertoli cell as a potential alternative to systemic immune suppression. Sertoli cells, when co-transplanted with therapeutic cells (for example pancreatic islets to treat diabetes), can provide both local immune suppression and trophic support, allowing the transplanted cells to survive indefinitely. This transplantation of Sertoli cells even works between species i.e. pig to rat. A considerable hurdle in bringing this type of therapy into the clinical domain is the availability of large numbers of well-characterised Sertoli cells.

Previous studies of rat Sertoli cell culture have reported that these cells spontaneously cease proliferation at the equivalent of day 10-12 post-partum, irrespective of the age of the donor animal. We have developed techniques for the isolation and culture of Sertoli cells which allows a highly pure preparation to proliferate in culture up to day 30 post-partum.

Sertoli cells are isolated from six-day-old rats by a process of enzymatic digestion followed by selective depletion of contaminating peritubular cells. Following this purification process, the level of contamination is less than 0.5%.

Proliferating Sertoli cells are highly responsive to follicle stimulating hormone (FSH), the classical Sertoli cell mitogen. We also find that Sertoli cell mitosis is modestly stimulated by activin, however the combination of activin and FSH reveals a striking synergy between these mitogens. The mechanism of this synergy will be discussed.

We have developed methods for differentiating these cells *in vitro* by using thyroid hormone, testosterone and retinoic acid. We show that these factors stimulate the expression of negative regulators of the cell cycle (p27 and p21?) and consequently suppress Sertoli cell division.

The application of these technologies to generate large numbers of Sertoli cells will aid both the study of Sertoli cell function, and the development of a clinically viable protocol that support cell based therapies. We are currently testing the applicability of our rat protocols to the culture of Sertoli cells from a primate model with the view to developing non-transformed lines of human Sertoli cells.

## ESTROGEN ELICITS LOBE SPECIFIC ACTIONS ON THE MATURE HYPOGONADAL PROSTATE AND SEMINAL VESICLES

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Estrogens have been shown to influence the developing and mature prostate, altering normal morphological and functional homeostasis. Estrogen action can be suppressed by the presence of other hormones, particularly androgens, and increased estrogen:androgen levels in aging human males are associated with prostatic pathogenesis. The administration of an estrogen stimulus in a wildtype environment alters the hypothalamic-pituitary-gonadal axis and the presence of androgens can modulate the estrogenic response. Therefore in this investigation we utilised the hypogonadal mouse (*hpg*), a gonadotropin-deficient model devoid of androgens and estrogens. This model was employed to investigate the direct effects of estrogen on the prostate and seminal vesicles (SV) in an environment free of other hormones.

*Hpg* mice were subcutaneously implanted with 17 $\beta$ -estradiol at 6wk. Anterior and ventral prostate lobes (AP; VP) and SV were microdissected from wildtype (WT), *hpg*, and estrogen-treated *hpg* mice at 12wk and prepared for stereological and immunohistochemical analysis (IHC).

The organ weights (AP, VP, SV) were significantly increased following estrogen-treatment in the *hpg* ( $P < 0.05$ ), yet never reestablished WT weights. Stereological analysis revealed uniform absolute volumetric increases in stromal, epithelial, and luminal compartments ( $P < 0.05$ ). Relative volumetric comparison demonstrated that the AP and SV, but not the VP, organ compartments were proportionally altered compared with the untreated *hpg*. The increased stromal volume was characterised by an increase in fibroblastic content in all organs, along with concomitant degeneration and disorganisation of the AP and SV smooth muscle layer. The epithelial changes were associated with intense basal cell proliferation and multilayering. The AP demonstrated squamous metaplasia and a significant inflammatory response characterised by local infiltration of neutrophils. In contrast, the VP showed transitional cell metaplasia of the epithelium, but no inflammation. These responses were shown to coincide with an upregulation of estrogen receptor-alpha and progesterone receptor immunoeexpression. No differences in the number of apoptotic cells were detected.

The induction of fibroblastic stroma at the expense of smooth muscle cell disruption in the estrogen-treated *hpg* AP and SV suggests the possibility of cellular dedifferentiation due to estrogen, reinforced by a lack of smooth muscle targeting by neutrophils and an absence of apoptosis in these cells. The changes in the stroma and epithelium are consistent with the presence of lobe-specific sensitivities between the AP and VP, particularly as evidenced by the alternative pathologies of the epithelia and smooth muscle, and also by the absence of inflammation in the VP. These data demonstrate that estrogens, in the absence of androgens, can induce abnormal mitogenic activity in the mouse prostate and SV, suggesting that androgens are required for normal prostate and SV growth.

## IDENTIFICATION OF NOVEL ANDROGEN REGULATED GENES IN THE PROSTATE

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The prostate is an androgen-dependent organ and the site of one of the most frequent cancers in men. Although androgen withdrawal causes temporary regression of prostate cancer through activation of androgen-repressible apoptotic mechanisms, this is inevitably followed by a relapse and progression of cancer due to proliferation of cells that are not dependent upon androgens for their survival. The genetic basis of this androgen-independent prostate cancer is presently unclear. The aim of this study therefore, was to identify genes expressed in the androgen deprived prostate. Such genes may be involved in the growth of prostate cancer cells that escape from the temporary restraint created by androgen ablation therapy. To achieve this goal, we have used the hypogonadal (*hpg*) mouse that is congenitally androgen deficient due to an intra-genetic deletion leading to an absence of testicular androgen secretion. As a result, the *hpg* mouse has infantile reproductive and sex accessory organs including the prostate. Using suppression subtractive hybridization between mRNA extracted from prostates of *hpg* and androgen-replaced *hpg* mice, we have cloned and characterized two novel genes— one of which was down (ADMP) and the other up-regulated (AUMP) by androgens.

ADMP expression was strong in untreated *hpg* but absent in androgen-replaced mouse prostates by RT-PCR analysis. Amongst various tissues, ADMP expression was strong in the prostate but weak in cortex, cerebellum and kidney. The full-length cDNA was cloned by 5'RACE and consisted of 880 nucleotides with an open reading frame (ORF) of 231 bp. Human EST and HTG database searches were conducted to identify the human homologue of ADMP. Sequence similarity between mouse and human ADMP at the ORF and amino acid level was 85.7% and 93.4% respectively. The predicted mouse and human protein of 76 amino acids show no secretory or nuclear localization signal but share sequence similarity with the yeast calcium-transporting ATPase 8 suggesting a possible role of ADMP in ion transport or regulation of intracellular calcium. Further study of human homologue of ADMP may reveal its possible involvement in prostate disease including hormone-refractory cancer.

AUMP gene was strongly expressed in androgen replete but absent in androgen deficient prostates as detected by RT-PCR. Full-length cDNA obtained by assembling sequences derived from 5' and 3' clones showed this gene to have a size of 805 bp with an ORF of 405 amino acids. AUMP showed selective expression in the prostate by RT-PCR and northern analysis of 8 mouse organs. In situ hybridisation localized AUMP expression to epithelial cytoplasm of the glandular duct in the androgen-sufficient prostate. Database searching revealed that AUMP shares partial sequence homology to palmitoyl protein thioesterase (PPT) indicating that AUMP may be a PPT-like protein operating selectively in androgen-sufficient prostate. However, its role in aberrant prostate growth remains to be clarified.

## ADRENAL GROWTH AND STEROIDOGENESIS ARE DIFFERENTIALLY REGULATED IN FETAL DEVELOPMENT.

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In most mammalian species, periods of active adrenal hyperplasia and steroidogenesis may be interrupted by periods of "quiescence" in which fetal adrenal gland is unresponsive to ACTH, adrenal growth is substantially slowed and steroidogenesis is suppressed. In the present study, we have investigated the developmental expression of key markers of adrenal growth and steroidogenesis. We have determined the expression of cyclin D1 (the rate limiting cyclin, which is activated during the progression of the G1 phase of the cell cycle and thus a marker of cellular hyperplasia), cytochrome P450 17 $\alpha$  hydroxylase (CYP17) and the melanocortin type 2 receptor (MC2R or ACTH receptor). Fetal sheep adrenal glands were collected from fetuses at 54 days of gestation (d) (n=8 pairs), 82-84d (n=5), 90-91d (n=5), 125d (n=5) and 140-145d (n=5). Northern blot analysis was performed using a 32P-labelled cDNA probes to human cyclin D1, ovine MC2R, bovine CYP17 and a 32P-labelled rat 18S rRNA oligo-probe was used to verify equal RNA loading. In fetal adrenals between 54d-145d, cyclin D1 mRNA was detected, with a major transcript size of 4.0kb and 2 minor bands at 6.0kb and 1.5kb. Adrenal expression of cyclin D1 mRNA:18S rRNA was significantly higher at 54d (7441.0  $\pm$  970.4) that at any later stage of gestation and fell by ~3 fold by 82d (2679.6  $\pm$  141.9) relatively low throughout the remainder of gestation (145d; 2070.5  $\pm$  334.4). Adrenal CYP17:18S was also high at 54d (527.0  $\pm$  102.3) and decreased 4 fold between 54d and 90d (140.6  $\pm$  31.3). In contrast to cyclin D1, CYP17:18S expression increased significantly by 125d (609.3  $\pm$  110.9), with a further 3 fold increase by 140-145d (2073.6  $\pm$  212.4). In fetal adrenals between 54d-145d, adrenal MC2R mRNA was detected, with a major transcript size of 3.5kb and 1 minor band at 1.5kb. Adrenal expression of MC2R:18S decreased between 54d (2338.9  $\pm$  74.6) and 90d (1242.8  $\pm$  237.6) and then increased by 125d (2295.47  $\pm$  187.94). In summary, we found that high expression of cyclin D1 occurs at ~50d gestation, at a time when there is rapid adrenal growth primarily due to cellular hyperplasia. The differential pattern of cyclin D1, CYP17 and MC2R expression throughout the phases of adrenal activation and quiescence suggests that cyclin D1 expression may not be regulated directly by ACTH. Further studies utilizing these specific markers of cellular hyperplasia and steroidogenesis may lead to the understanding of underlying the intracellular mechanisms which lead to the dissociation between adrenal growth and steroidogenesis.

## PROGESTERONE RECEPTOR EXPRESSION AND LOCALIZATION IN LABORING AND NON-LABORING HUMAN FETAL MEMBRANES

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The fetal membranes (amnion and chorion) play central roles in the parturition process by producing prostaglandins (PGs), potent stimulators of uterine contraction. PG production by the fetal membranes is inhibited by progesterone suggesting that the fetal membranes are targets for progesterone action. In all species, progesterone is required for the maintenance of pregnancy and in most species the initiation of parturition is associated with a dramatic fall in circulating progesterone levels (progesterone withdrawal). In the human, progesterone withdrawal may also play a role in the initiation of human parturition, however, the mechanism by which this is achieved is unclear since circulating progesterone levels remain elevated throughout labor and delivery. To explain this conundrum, we hypothesized that progesterone withdrawal in human parturition is mediated by changes in target tissue responsiveness to circulating progesterone via alterations in progesterone receptor (PR) expression. Two human PR isoforms have been identified: PRA and PRB. In general, PRB functions as the ligand-activated transcriptional regulator of progesterone responsive genes, whereas PRA suppresses actions mediated through PRB. Thus, PR-B mediates progesterone action, whereas PR-A is an endogenous inhibitor of progesterone action. Based on these observations we hypothesized that progesterone withdrawal in human parturition is mediated by an increase in PRA:PRB ratio. To test this hypothesis we examined the localization and extent of expression of PR-A and PR-B in term human amnion and chorion obtained from scheduled cesarean section deliveries before the onset of labor (not in labor group; n=10) or after normal spontaneous vaginal deliveries (in labor group; n=6). Localization of PRs was determined by immunohistochemistry using antibodies specific for each isoform. Abundance of mRNA transcripts encoding PRs was determined by real time quantitative RT-PCR and normalized to 18S rRNA. Moderate PR-A and PR-B immunostaining was detected mainly in nuclei of chorionic trophoblast. Only weak nuclear staining for PR-A and PR-B was detected in amnion cells. Sparse and generally very weak staining for PR-A and PR-B was detected in cells within the connective tissue layers separating the amnion and chorion and within the decidua. There was no apparent change in PR immuno-localization in relation to labor status. Consistent with the localization data, relative abundance of mRNAs encoding PR-A and PR-B were 30- to 50-fold higher in chorion compared with amnion. In both tissues, abundance of mRNA encoding PR-A was 3 to 4-fold greater than that encoding PR-B. Levels of mRNA transcripts encoding PRs were not altered in either tissue by labor status. These data indicate that chorionic trophoblasts are the principal target for progesterone action in the human fetal membranes and are consistent with the chorion being an important site of progesterone-regulated PG metabolism.

## THE IN VIVO CONTROL OF PROSTAGLANDIN H SYNTHASE -2 (PGHS-2) GENE EXPRESSION IN THE HUMAN AMNION AT PARTURITION.

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Prostaglandins (PGs) produced in the amnion membrane play a pivotal role in the initiation and maintenance of labor in humans. The activity of prostaglandin endoperoxide H synthase (PGHS), which catalyses the committing and rate limiting step of PG biosynthesis, increases in the amnion before and during labour resulting in enhanced PG production. The increased PGHS activity is the consequence of the selective induction of the mRNA encoding the PGHS-2 isoform. While the up-regulation of amniotic PGHS-2 mRNA, protein and activity at labour is well documented and its importance in the physiology of human parturition is widely recognized, the in vivo mechanism of the induction process is unknown. Therefore, our aim in the present study was to determine the mechanisms that control PGHS-2 gene expression in the amnion at parturition.

PGHS-2 gene transcription rates were measured by transcriptional run-on assays in cell nuclei isolated from human amnion immediately after spontaneous labour (SL) or elective Caesarean section (CS) at term. Total RNA was also isolated and the levels of PGHS-2 mRNA and heterogenous nuclear RNA (hnRNA), the immediate product of PGHS-2 gene transcription and a surrogate measure of gene transcription rate) were determined using quantitative real time reverse transcriptase polymerase chain reaction (RT-PCR), with exon spanning and intron specific primers, respectively. PGHS-2 mRNA stability and hnRNA processing rates were determined by RT-PCR after incubating amnion tissue in the presence of the transcription inhibitor DRB for up to 24 h. Results were normalized to  $\beta$ -Actin transcription rate or mRNA abundance as appropriate.

PGHS-2 run-on transcription rates correlated significantly ( $p < 0.05$ , parametric and Spearman analyses,  $n = 11$ ) with PGHS-2 mRNA levels. Further, PGHS-2 mRNA abundance showed significant correlation with PGHS-2 hnRNA abundance both in CS ( $p < 0.02$ ,  $n = 15$ ) and SL ( $p < 0.001$ ,  $n = 13$ ) amnions. PGHS-2 hnRNA displayed fast processing rate (half life:  $22.01 \pm 1.42$  min and  $20.17 \pm 1.07$  min at CS and SL, respectively; mean  $\pm$  SE,  $n = 3$ ). In contrast, the mature PGHS-2 mRNA was highly stable with a half-life of  $71.7 \pm 21.99$  h at CS and  $44.9 \pm 3.57$  h at SL (mean  $\pm$  SE,  $n = 3$ , not significantly different by t-test). Incubation of amnion explants for up to 24 h without transcription inhibitor resulted in a 90% drop of hnRNA abundance within 2 hours. PGHS-2 mRNA, however, showed a gradual accumulation to more than 200% ( $p < 0.05$ , one-way ANOVA) of the zero h value over an 24 hour period.

These results provide a coherent picture of PGHS-2 regulation at birth. Thus, PGHS-2 gene transcription rate is the primary factor determining PGHS-2 mRNA abundance in the human amnion before and after labour. PGHS-2 transcription is acutely up-regulated by unknown endocrine/paracrine or possibly autocrine factor(s). The expressed PGHS-2 mRNA is highly stable, allowing for the continual and robust accumulation of PGHS-2, which over days or weeks may reach a threshold sufficient to induce labour. The regulatory factors and transcriptional mechanisms involved in this process remain to be established in order find suitable targets for intervention to block PGHS-2 induction if early birth needs to be delayed. Supported by RMC, University of Newcastle

## PERICONCEPTIONAL NUTRIENT RESTRICTION AND PROGRAMMING OF FETAL BLOOD PRESSURE AND HYPOTHALAMO-PITUITARY ADRENAL (HPA) AXIS DEVELOPMENT

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Intrauterine growth restriction is associated with an increased prevalence of cardiovascular disease, non-insulin dependent diabetes mellitus and the Metabolic Syndrome in adult life. It has been postulated that decreased maternal nutrient intake alters the development of the hypothalamo-pituitary-adrenal (HPA) axis and that reprogramming of this axis may play a role in the programming of adult disease. We have investigated the separate and combined effects of restriction of maternal nutrient intake during either the periconceptional period or after the first week of gestation on fetal arterial blood pressure and HPA axis development. Fifty two ewes were used in this study. Prior to mating, ewes were randomly assigned to one of two feeding regimes, Control (C, n = 23) or Restricted (70% of the control allowance, R, n=29). After a minimum period of 60 days, ewes were mated and 7 days after mating, ewes from each feeding regime were then assigned to the C (C-C and R-C) or R (C-R and R-R) plane of nutrition for the remainder of gestation (until delivery or postmortem after 140d gestation). Fetal and maternal blood samples were collected from every 2-3d from 110d gestation until delivery or postmortem. Basal fetal arterial blood pressure and the fetal blood pressure response to the Angiotensin Converting Enzyme (ACE) inhibitor captopril, were measured between 115-125d and 135-145d gestation. Plasma glucose concentrations were lower ( $p<0.05$ ) in twin compared to singleton fetuses in all nutritional groups. In both singletons and twins, plasma glucose concentrations were lower ( $p<0.01$ ) in the C-R and R-R groups than in the C-C and R-C groups. Fetal arterial blood pressure was significantly higher in twin fetal sheep which were undernourished in the periconceptional period (R-C and R-R; 115-125d:  $41.4 \pm 1.2$  mmHg, 135-145d:  $50.5 \pm 2.2$  mmHg) when compared to fetal sheep in the control group during the periconceptional period (C-R and C-C; 115-125d:  $38.5 \pm 1.6$  mmHg, 135-145d:  $42.5 \pm 1.9$  mmHg). In singleton fetal sheep, there was no effect of restricted periconceptional nutrition on the fetal blood pressure response to captopril. Restricted gestational nutrition however, resulted in a significantly smaller reduction in fetal mean arterial blood pressure in response to the captopril infusion compared to control gestational nutrition. In twin fetal sheep, there was no significant effect of periconceptional or gestational nutrition on the fetal mean arterial blood pressure responses to captopril. In twin fetuses, plasma ACTH, but not cortisol, concentrations were higher ( $p<0.05$ ) throughout late gestation in animals which were undernourished in the early periconceptional period (R-C and R-R) when compared with the C-R and C-C groups. These data indicate that undernutrition before and in the first week after conception has a significant impact on the development of fetal blood pressure and the HPA axis in twin fetuses.

## FETAL GROWTH RESTRICTION ALTERS INSULIN SENSITIVITY OF GLUCOSE METABOLISM DIFFERENTIALLY WITH GENDER IN THE ADULT GUINEA PIG.

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Epidemiological studies show that fetal growth restriction (FGR), as indicated by being light, short or thin at birth, is associated with insulin resistance and related diseases, such as glucose intolerance and type 2 diabetes, in mature adult men and women. We have shown that insulin resistance is present in young men but not in young women, aged in their twenties, who were thin at birth, suggesting gender differences in the timing of onset of prenatally induced insulin resistance. We therefore hypothesised that FGR due to increased litter size, would be associated with greater adult insulin resistance of glucose metabolism (as occurs in type 2 diabetes generally), in male compared to female guinea pigs.

The basal and insulin-stimulated rates of whole body glucose utilisation and its major components, peripheral glucose utilisation and glucose production, were measured using concomitant 3-[<sup>3</sup>H]glucose infusion prior to and during a hyperinsulinaemic euglycaemic clamp (HEC), in young adult guinea pigs. The partitioning of glucose utilisation into glycolysis or storage under was also determined. Insulin sensitivity of whole glucose metabolism, as indicated by the steady state glucose infusion rate achieved during HEC decreased with birth weight overall ( $r=0.55$ ,  $p<0.0005$ ,  $n=36$ ) and when males ( $r=0.60$ ,  $p<0.005$ ,  $n=20$ ) and females ( $r=0.50$ ,  $p<0.025$ ,  $n=16$ ) were considered separately. The rate of decrease in insulin sensitivity of glucose metabolism with birth weight did not vary with gender.

The basal rate of whole body glucose utilisation, production, glycolysis or storage did not vary with birth weight overall ( $n=19$ ) or with gender. Insulin sensitivity of whole body glucose utilisation also decreased with birth weight ( $r=0.69$ ,  $p<0.0005$ ,  $n=19$ ) overall, and in males ( $r=0.88$ ,  $p<0.001$ ,  $n=10$ ) and females ( $r=0.56$ ,  $p<0.06$ ,  $n=9$ ) separately. The rate of decrease in insulin sensitivity of glucose utilisation with birth weight was greater in males ( $0.0034 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} / \mu\text{U} \cdot \text{ml}^{-1}$  per gram birth weight) compared to females ( $0.0026 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} / \mu\text{U} \cdot \text{ml}^{-1}$  per gram birth weight) ( $p<0.002$ ). The insulin sensitivity of glucose production decreased with decreasing birth weight ( $r=0.42$ ,  $p<0.05$ ,  $n=19$ ) overall and in males ( $r=0.83$ ,  $p<0.001$ ,  $n=10$ ) but not in females (ns). The insulin sensitivity of glycolysis did not vary with birth weight overall or with genders. The insulin sensitivity of whole body glucose storage decreased with birth weight ( $r=0.60$ ,  $p<0.005$ ,  $n=19$ ) overall and in males ( $r=0.65$ ,  $p<0.02$ ,  $n=10$ ) and females ( $r=0.66$ ,  $p<0.05$ ,  $n=9$ ) separately. The rate of decrease in insulin sensitivity of glucose storage with decreasing birth weight was greater in females ( $0.0065 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} / \text{mU} \cdot \text{ml}^{-1}$  per gram birth weight) than in males ( $0.0026 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} / \text{mU} \cdot \text{ml}^{-1}$  per gram birth weight) ( $p<0.0005$ ). In conclusion, FGR substantially impairs whole body insulin sensitivity in young adult guinea pigs in a gender specific manner. The variation in both adult peripheral and hepatic insulin sensitivity accounted for by birth weight, and their decline with birth weight is greater in males than in females.



## PRENATAL PROGRAMMING OF IMPAIRED GLUCOSE HOMEOSTASIS IN THE ADULT GUINEA PIG

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In recent years, epidemiological studies have demonstrated that impaired growth in early life, as indicated by being light or thin at birth, is associated with impaired glucoregulation and Type 2 diabetes in adult life. Experimental restriction of maternal feed or protein intake, mostly in the rat, usually does not alter or even improves insulin sensitivity and glucose tolerance in adult offspring, however. In contrast, we have observed in the guinea pig, which is more mature at birth than the rat, that spontaneous fetal growth restriction is associated with substantial whole body and peripheral insulin resistance in the young adult, although normal glucose tolerance, due to compensatory hyperinsulinaemia. The aim of the current study was to determine whether the insulin resistant young adult guinea pig of low birth weight would subsequently develop impaired glucose homeostasis with increasing age, as occurs in humans.

Pregnant guinea pigs were allowed to deliver and size at birth was measured in offspring, which then had vascular catheters inserted into the carotid artery and jugular vein under general anaesthesia and strict asepsis at 400 days of age. An intravenous glucose tolerance test (IVGTT) was performed in these chronically catheterised older adult guinea pigs ( $419 \pm 3$  days) ( $n=30$ ). Arterial blood was sampled prior to and following the intravenous administration of a 0.5g/kg body weight bolus dose of dextrose (50% w/v), in order to determine the fasting blood glucose concentration and glucose tolerance respectively. Glucose tolerance was calculated as the area under the glucose concentration curve (GAUC).

Adult fasting blood glucose concentrations increased with decreasing weight ( $r=-0.347$ ,  $p=0.03$ ,  $n=30$ ) and abdominal circumference ( $r=-0.357$ ,  $p=0.027$ ,  $n=30$ ) at birth. When males and females were analysed separately however, fasting blood glucose concentrations increased with decreasing weight ( $r=-0.695$ ,  $p=0.001$ ,  $n=16$ ) and abdominal circumference ( $r=-0.528$ ,  $p=0.018$ ,  $n=16$ ) at birth in females, but not in males. Glucose tolerance also decreased with decreasing weight at birth ( $r=-0.332$ ,  $p=0.046$ ,  $n=27$ ), and those animals in the lowest birth weight tertile exhibited an increased GAUC (+54%), when compared to those in the highest birth weight tertile ( $p<0.05$ ). When genders were analysed separately, GAUC increased with decreasing birth weight in females ( $r=-0.45$ ,  $p=0.046$ ,  $n=15$ ), but was not associated with any parameter of size at birth in males. In conclusion, spontaneous fetal growth restriction alters adult glucose homeostasis in the older guinea pig in a gender-specific manner, producing female offspring who are diabetic (hyperglycaemic and glucose intolerant) and male offspring with unaltered fasting glucose levels and glucose tolerance.

## PROGESTERONE RECEPTOR (PR) EXPRESSION IN THE PREGNANT HUMAN MYOMETRIUM: EVIDENCE FOR LOCAL PROGESTERONE WITHDRAWAL VIA INCREASED EXPRESSION OF PR-A

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Progesterone promotes myometrial quiescence and is essential for establishment and maintenance of the pregnancy. Consistent with this action, parturition in most mammals is associated with decreased circulating progesterone (progesterone withdrawal). Despite the strong causal association between progesterone withdrawal and the initiation of human parturition, circulating progesterone levels do not decrease during labor and delivery. To explain this conundrum we hypothesize that progesterone withdrawal in human parturition is not controlled by changes in circulating progesterone levels but instead is mediated by changes in target tissue responsiveness. Target tissue responsiveness to progesterone is principally determined by the abundance and type of cognate receptors. The human progesterone receptor (PR) exist as two major subtypes: PR-A and PR-B. PR-B is the principal ligand-dependent transcriptional activator of progesterone responsive genes, whereas PR-A represses the transcriptional activity of PR-B. Based on these observations we reasoned that progesterone withdrawal in human pregnancy could occur locally in myometrial cells by increased expression of PR-A relative to PR-B. Therefore, we examined the extent of expression (assessed by abundance of specific mRNAs measured by real-time quantitative RT-PCR) of PR-A and -B in term (37-42 weeks) human uterus collected from women who gave birth by scheduled cesarean section before the onset of labor (not in labor group;  $n=6$ ) or after emergency cesarean section during labor (in labor group;  $n=6$ ). To examine the relationship between PRs and other parturition-associated genes, we also determined the extent of expression of the estrogen receptors  $\alpha$  and  $\beta$  (ER $\alpha$  and ER $\beta$ ) and prostaglandin endoperoxide synthase type -II (PGHS-II). Relative abundances (normalized to 18S rRNA) of mRNAs encoding PR-A, ER $\alpha$ , and PGHS-2 and the proportion of PR-A relative to PR-B (PR-A/PR-B) were significantly ( $P<0.05$ ) increased in laboring myometria. There was no change in abundance of mRNAs encoding PR-B and ER $\beta$ . A significant positive correlation between ER $\alpha$  mRNA and the PR-A/PR-B ratio ( $r^2=0.8621$ ;  $P<0.01$ ) was detected in the non-laboring group but not in the laboring group. Similarly, PGHS-2 mRNA levels also were closely correlated with the PR-A/PR-B ratio ( $r^2 = 0.7730$ ;  $P<0.05$ ) in non-laboring specimens. There was no correlation between ER $\beta$ , PRs and PGHS-2 before or during labor. These data suggest that human parturition involves increased expression of PR-A, ER $\alpha$  and PGHS-2 in the uterus and that prior to the onset of labor, the gradual increase in PR-A expression relative to PR-B is associated with increased expression of ER $\alpha$  and PGHS-2. These findings support the hypothesis that progesterone withdrawal in human parturition is mediated by increased expression of PR-A. This event may be related to increased estrogen responsiveness via increased expression of ER $\alpha$  and increased local prostaglandin activity via increased expression of PGHS-II. In the non-pregnant uterus progesterone inhibits ER expression. We propose that this inhibition also occurs in the pregnant uterus and that the increased expression of ER $\alpha$  is due to local progesterone withdrawal via increased expression of PR-A. As estrogen is thought to increase expression of contraction associated genes in the uterus, this reciprocal relationship between ER $\alpha$  and PR-A/PR-B in myometrium may be critical for pregnancy maintenance and parturition.

## PARATHYROID HORMONE-RELATED PROTEIN CONCENTRATIONS IN MATERNAL PLASMA AND AMNIOTIC FLUID ARE INCREASED IN HUMAN MACROSOMIC AND DIABETIC PREGNANCIES

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Parathyroid hormone-related protein (PTHrP) acts as a growth factor to promote cellular growth and differentiation, stimulate placental calcium transport and relax uteroplacental vessels. PTHrP is circulating in low concentrations in maternal and umbilical cord plasma (2) and is present in high concentrations in amniotic fluid during late pregnancy (1,2). Macrosomic (birth weight greater than 90<sup>th</sup> percentile for gestational age) and gestational diabetic pregnancies are associated with significant perinatal risks. Our aim was to measure PTHrP (by specific N-terminal radioimmunoassay), calcium, glucose and lactate concentrations in maternal and umbilical vein plasma and amniotic fluid from term ( $\geq 37$  weeks) pregnancies. Samples were collected from women following normal pregnancies delivered by caesarian section (CS) (Normal CS) and normal vaginal delivery (NVD) (Normal NVD) and complicated pregnancies including macrosomia (Macro), non-insulin dependent (diet controlled) gestational diabetes mellitus (GDM NID) and insulin dependent gestational diabetes (GDM ID). Data were analysed by analysis of variance.

Gestational age at delivery and birth and placental weights were not different between the normal and GDM groups but were significantly elevated in the Macro group compared to all other groups ( $p < 0.05$ ). There were no significant differences in immunoreactive PTHrP concentrations in cord venous plasma between the groups. Maternal plasma and amniotic fluid PTHrP concentrations were significantly greater in GDM NID, but not GDM ID, pregnancies compared to Normal (CS and NVD) ( $p < 0.05$ ). These changes were not associated with alterations in plasma ionic calcium, glucose or lactate concentrations. Amniotic fluid PTHrP concentrations were significantly elevated in Macro pregnancies compared to Normal (CS and NVD) ( $p < 0.05$ ). Cord venous calcium concentrations were significantly lower in GDM NID and GDM ID compared to Normal and Macro pregnancies ( $p < 0.05$ ).

In conclusion, Macrosomic and GDM NID, but not GDM ID, pregnancies are associated with altered PTHrP expression in maternal plasma and amniotic fluid. The effects of insulin on PTHrP expression and the effects of these on fetal growth, cellular differentiation and perinatal morbidity and mortality remain to be established.

## A RANDOMISED PLACEBO CONTROLLED CLINICAL TRIAL OF RECOMBINANT HCG ON INSULIN SENSITIVITY, MOOD AND QUALITY OF LIFE IN OLDER MEN.

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Longitudinal studies now confirm that increasing age is associated with a fall in serum testosterone and an increase in insulin resistance and diabetes mellitus. Nested case control studies have shown that low serum testosterone is associated with increased insulin resistance and can predict development of diabetes mellitus. Androgen therapy in middle aged abdominally obese men may reduce insulin resistance but whether such therapy is effective in older men is not known although any improvement could have major public health implications. HCG (human chorionic gonadotropin) can be conveniently self-administered and may produce effects from testicular secretion of estradiol and other steroids as well testosterone. We evaluated the safety and efficacy of 3 months treatment with subcutaneous recombinant hCG (Ovidrel) on insulin sensitivity, mood and quality of life in ambulant, community dwelling men  $> 60$  years of age with partial androgen deficiency ( $T \leq 15$  nmol/L on two occasions). 40 eligible men were randomised to receive recombinant hCG 250 mcg twice each week ( $n=20$ ) or placebo ( $n=20$ ) injections and were studied before, monthly during and one month after the treatment period. All 40 men (mean age 67 (range 60 – 85)) completed the study and groups were well matched for all baseline variables (including age, height, weight or insulin sensitivity (Homeostasis Model, HOMA)). Insulin sensitivity was also assessed by euglycaemic hyperinsulinaemic clamp at baseline and at the end of the treatment period in the first 30 consecutive men who did not have known diabetes mellitus. 17 of these men received placebo. Treatment and placebo groups were compared by repeated measures ANOVA as changes from baseline. Significant expected increases were seen in total (10 nmol/L) and free testosterone (230 pmol/L) and estradiol (180 pmol/L) in conjunction with suppression of gonadotropins ( $P < 0.001$  for each). No significant change was seen in insulin resistance (HOMA and euglycaemic clamp) or beta cell function (HOMA) even when adjusted by lean body mass or diabetes mellitus despite a significant increase in lean body mass (3 kg,  $P < 0.001$ , Lukaski bioimpedance). Subcutaneous fat (skinfold measurements), abdominal girth and serum leptin all decreased, but not significantly. HCG treatment did not alter any dimension of mood or quality of life, but a significant reduction in total mood disturbance as well as confusion and tension-anxiety (Profile of Mood States) and bodily pain (SF36) ( $P < 0.05$  for each) was detected in the placebo group. We conclude that 3 months treatment with recombinant hCG demonstrates expected hormonal effects, but had no effect on insulin sensitivity despite suggestive changes in body composition.

## ANDROGENETIC ALOPECIA DIAGNOSED IN A PATIENT WITH COMPLETE ANDROGEN INSENSITIVITY SYNDROME.

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Androgenetic alopecia (AGA) is a polygenic disorder which is thought to be dependent on the presence of circulating androgens and a functional androgen receptor (AR). Dihydrotestosterone (DHT) is the androgen critical for the development of this progressive patterned hair loss as 5 $\alpha$ -reductase deficient individuals unable to synthesize DHT fail to develop baldness even where there is a clear well-defined familial history.

We present a patient diagnosed with complete androgen insensitivity syndrome (CAIS), with no evidence of androgen activity who also exhibited early stage female pattern AGA. We investigated this individual in an attempt to explain this paradox. After re-presenting with a diagnosis of AIS at the age of 40, a bilateral orchidectomy was performed. Approximately 3 years later the patient was diagnosed with early stage female pattern AGA. This was confirmed on multiple histological examinations. No clinical or biochemical evidence for an alternative cause of hair loss was found. Despite the diagnosis of AIS the patient was treated with spironolactone, an AR antagonist. There was cessation of any further hair loss and an improvement in the hair quality was observed. Patterned hair loss observed in patients with AGA is different in men and women. Characteristically, women have diffuse thinning over the crown but the frontal hairline is preserved. Histological investigation revealed a decrease in the terminal to vellus hair ratio from >7:1 to <3:1 and an increase in the number of hairs in the telogen resting phase from <7% to >16% typical of AGA.

To explain why an individual with CAIS and no androgen activity could develop AGA, a condition thought to be dependent on androgen activity, we have investigated the AR gene in this subject. Sequence analysis was performed on each exon of the gene. Analysis of exon 1 revealed a single base deletion of a cytosine base at position 1515 causing a frameshift in the downstream sequence resulting in the introduction of a stop codon at amino acid 508. This truncation of the AR explains the subject's CAIS phenotype. Two additional mutations were identified in exon 1 and 4 respectively. This unique CAIS individual allows us to propose that the AR may not be necessary for the development of AGA as in this case there is no functioning AR. It may be that small amounts of androgens could work independently of the AR by some other as yet unidentified pathway. The apparent beneficial effect of spironolactone remains to be explained.

## DOES ANTIBIOTIC IMPREGNATION REDUCE THE RATE OF TESTOSTERONE PELLET EXTRUSION?

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Testosterone pellet implantation is a safe and convenient form of long-term androgen replacement. Occasional extrusion of one or more pellets in the months following implantation remains the most frequent adverse effect. The objective of this study was to determine whether extrusion could be reduced by adjuvant antibiotic treatment of the implants at the time of implantation. The hypothesis was that extrusion may be related to a slow-growing insidious pathogen introduced at implantation causing extrusion. We conducted a prospective, randomised, parallel-group, open-label study in a single center to determine whether antibiotic soaking prior to implantation could reduce the rate of extrusion. Accordingly, androgen deficient men requiring routine testosterone implantation were randomised into one of 2 groups. One group had their pellets soaked for ~2 minutes in gentamicin 80 mg in 2 mls sterile water prior to implantation, the control group had no modification to the routine implantation procedure. The primary end-point of the study, extrusion rate per procedure, and the secondary end-point, post-procedural infection were evaluated prospectively by self-report from the participants, and verified when they returned for subsequent implantation following 400 implantation procedures involving 186 androgen deficient men. Other procedure related information (site used, skin preparation etc) which may influence extrusion rate was collected at implantation. The extrusion rate was not statistically different between the two groups: extrusion rate 24/205 (11.1%) for the control group vs 17/195 (8.7%) for the antibiotic soak group (p=0.2). Extrusion was not related to 14 different batch numbers used in the study (p=0.15), nor to shaving the site or not (p=0.32), or use of an old vs new site (p=0.59). Multiple extrusions of pellets from the same implant procedure occurred almost as frequently as single pellet extrusions (19/41  $\geq$ 1 vs 22/41 single). Extrusions were no more likely after overt suppuration 18/400 (4.5%) than where none was evident 23/400 (5.7%) (p=0.42). One operator experienced more total extrusions (p=0.0002) and more infection related extrusions (p=0.0008) but not bruising (p=0.06) than others. We conclude that an antibiotic soak prior to implantation does not decrease extrusion rate of testosterone pellets. Neither topical preparation nor re-use of old sites influences or infection but there are potentially significant between-operator difference which cannot be attributed to either operator experience or implantation style. Further studies to reduce extrusion rate examining procedure-related events are warranted.

## THE OPTIMIZATION OF CHROMATOGRAPHIC SEPARATION OF ESTROGENS AND PROGESTERONE DERIVATIVES USING TEMPERATURE-DEPENDENT INCLUSION CHROMATOGRAPHY

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The great diversity of estrogens and progesterone derivative structures and their wide range of polarities present special problems for the simultaneous analysis of both classes of steroids in one sample using classical high-performance liquid chromatographic methods (HPLC) [1-3]. This study reports a simple strategy for the optimization of HPLC separation of steroids using temperature as the critical parameter for selectivity when the mobile phase is modified with an inclusion agent. As model compounds six estrogenic steroids (estetrol, estriol, 17 $\beta$ -estradiol, 17 $\alpha$ -estradiol, estrone, equilin) and two progesterone derivatives (17 $\alpha$ -hydroxyprogesterone and 20 $\alpha$ -hydroxyprogesterone) were chosen. All steroids were chromatographed using a range of column temperatures from 0 to 80°C and the mobile phase either unmodified as modified with the addition of  $\beta$ -cyclodextrin.

The retention of the steroids investigated is strongly influenced by temperature when the mobile phase is modified with  $\beta$ -cyclodextrin. Particularly, for 17 $\beta$ -estradiol and 20 $\alpha$ -hydroxyprogesterone a strong deviation from linear Van't Hoff plots and a remarkable affinity for  $\beta$ -cyclodextrin have been observed. The experimental data indicate that retention of inclusion complexes can be varied between two lines formed by the Van't Hoff plot of the  $\beta$ -cyclodextrin and the Van't Hoff plot of the uncomplexed solute. In addition, we demonstrate that the retention of an inclusion modifier plays an essential role in the chromatographic behavior of the solutes investigated.

From a practical point of view, the temperature changes produce significant differences in the separation of steroids. Using third-order polynomial equations, minimum separation factor values ( $\alpha_{min}$ ) were calculated for temperatures from -10 to +100°C with 1°C steps. The best selectivity was observed at the temperatures 47°C and 26°C. For both column temperatures excellent separation of an eight component mixture was obtained. Because the optimum separation for multicomponent mixtures is often a compromise between maximum separation and minimum analysis time, the temperature of 26°C can be recommended for simultaneous analysis of the investigated steroids in biological samples.

## MECHANISMS OF GROWTH INHIBITION IN MCF-7 BREAST CANCER CELLS BY A PURE OESTROGEN ANTAGONIST

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The molecular mechanisms underlying antioestrogen-mediated inhibition of cell cycle progression are not fully defined. Previously we showed that the pure antioestrogen ICI 182780 (Faslodex) could decrease the expression of the cell cycle regulatory gene, cyclin D1, resulting in inactivation of cyclin D1/Cdk4 activity and subsequently decreased phosphorylation of the retinoblastoma protein (pRb). We now show that cyclin E/Cdk2 activity also decreases following ICI 182780 treatment and that this change occurs prior to changes in S phase progression. The inhibition of cyclin E/Cdk2 activity results from a shift of the CDK inhibitor p21 from cyclin D1/Cdk4 to cyclin E/Cdk2. Antisense oligonucleotides to cyclin D1 could mimic the initial events seen following antioestrogen treatment and inhibition of p21 with antisense oligonucleotides could attenuate the growth inhibitory effects of ICI 182780 highlighting the importance of decreased cyclin D1 and the shift of p21. However, these events are preceded by a decrease in the protein level of the proto-oncogene c-Myc, one of the first detectable changes following antioestrogen treatment. Antisense inhibition of c-Myc could mimic many of the later events seen after antioestrogen treatment, such as loss of cyclin D1 protein, a shift of p21 from cyclin D1/Cdk4 into cyclin E/Cdk2 and inhibition of cyclin E/Cdk2 activity, implicating decreased c-Myc expression as a critical early event in antioestrogen-mediated growth inhibition.

Antioestrogen treatment also led to molecular changes characteristic of growth arrest in quiescence (G<sub>0</sub>) as opposed to growth arrest in G<sub>1</sub>, including hyperphosphorylation of the E2F4 transcription factor and association of E2F4 with the pocket protein, p130. This result was specific to pure antioestrogens and was not shared by tamoxifen-like antioestrogens. These data have implications for the ability of antioestrogen-arrested cells to re-enter the cell cycle in response to diverse mitogenic stimuli.

## THE EFFECT OF REDUCED IGF-II/M6P RECEPTOR ON TUMORIGENESIS OF MDA-MB-231 BREAST CANCER CELLS.

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Loss of heterozygosity with accompanying mutations and deletions in the Insulin-like growth factor-II/Mannose 6-phosphate (IGF-II/M6P) receptor gene in a variety of tumour types suggests the IGF-II/M6P receptor is a tumour suppressor. It is believed to act by binding and degrading the potent mitogen IGF-II, thereby regulating IGF-II mediated growth via the IGF-I receptor. The IGF-II/M6P receptor is also involved in the activation of latent form of TGF- $\beta$  and the transport of lysosomal enzymes such as Cathepsin D from Golgi to the endosomes. To examine the effect of reduced expression of IGF-II/M6P receptor, MDA-MB-231 breast cancer cells were transfected with IGF-II/M6P receptor antisense cDNA. Clones were isolated and screened for membrane IGF-II/M6P receptor levels by western blot analysis and by IGF-II/M6P receptor ELISA. No effect on receptor expression was observed in cells transfected with a sense cDNA construct, hence these cells were used as controls. Two sense and antisense cDNA transfected cell lines (50% and 60% reduction in IGF-II/M6P levels) were selected to examine the effect on growth and tumorigenicity of the IGF-II/M6P transfectants *in vivo*. Cells (107/200  $\square$ ) were injected subcutaneously at the dorsal neck of athymic (nude) mice, tumours were measured weekly and wet tumour weights determined at 9-12 weeks. Development of tumours were more frequent in mice injected with antisense transfected cells displaying reduced receptor levels (79%) compared with those injected with sense cDNA transfected cells (44%) as shown in Table 1. Moreover, antisense cDNA transfected cells developed into significantly larger tumours than the sense cDNA controls by ANOVA. Despite the differences in their ability to grow *in vivo*, there was no significant difference in *in vitro* growth rate. As hypothesized, the antisense cDNA transfected cells showed a 2-10 fold decrease in uptake of [<sup>125</sup>I]IGF-II over 2 hours at 37  $\square$ C. Further investigation of the IGF axis in the antisense cDNA transfected cells by western ligand blot analysis also revealed about 10-14 fold increase in the secreted levels of IGF binding protein 1 (IGFBP-1) (S:5.54ng/ml, AS:78.22ng/ml by radioimmunoassay) and 7-10 fold increase in IGFBP-4 by immunoblot. No change in cathepsin D, total and active TGF- $\beta$  levels were observed in medium conditioned by these cells. Taken together, the reduction in IGF-II/M6P seems to play an important role in enhancing growth and tumorigenicity of breast cancer cells. The relationship between the altered IGF-II/M6P receptor and marked increase in the IGFBPs are currently being studied. The mechanism of action of this receptor involved in modulating tumour growth is also under investigation.

Table 1. **Tumour growth in nude mice**

Transfected Cell line	Number of mice	Tumour Take rate (%)	Wet tumour Mass (g)
IGF-II/M6P Sense	31	44	0.026
IGF-II/M6P Antisense	49	79**	0.246*

\*\*p<0.0001, by Chi squared test. \*p<0.05 by ANOVA

Supported by NH & MRC.

## NOVEL EGF-RECEPTOR MRNA-PROTEIN INTERACTIONS: POTENTIAL TARGETS FOR THERAPEUTICS IN BREAST CANCER

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The epidermal growth factor-receptor (EGF-R) plays an important role in the growth and progression of oestrogen receptor negative (ER-ve) human breast cancers. EGF binds with high affinity to the EGF-R and activates a variety of second messenger pathways that impact on cellular proliferation. Thus, the EGF-R is an excellent target for anti-tumour therapy. However, current strategies, which target either the EGF-R extracellular or tyrosine kinase domain with antibodies, produce clinical improvement in only a relatively small proportion of patients. Improved understanding of the mechanisms regulating EGF-R expression may provide novel intracellular targets for therapy. In this regard, RNA-protein interactions have become important potential targets for novel therapeutics. We have shown recently that mRNA decay plays a central role in the regulation of EGF-R expression in breast cancer cells<sup>1</sup>. We have identified a novel EGF-regulated ~260 nt. *cis*-acting element in the 3'-untranslated region (3'-UTR) of EGF-R mRNA which contains two distinct AU-rich sequences (~75 nt.), EGF-R1A and EGF-R2A. RNA gel-shift identified cytoplasmic proteins (~55-80 kD) from breast cancer cells that bound specifically to the *cis*-acting element, and whose binding activity was rapidly down regulated by EGF and phorbol esters. Using the EGF-R2A probe as bait in yeast three-hybrid screening of a human breast cancer cDNA library, we identified several novel EGF-R RNA-binding proteins. One belongs to a family of bifunctional signalling molecules that has the capacity to bind mRNA, through a novel RNA-binding domain, and growth factor receptors via a protein-protein interaction domain. The functional role of this protein has been extensively characterised in cells and nude mice. Overexpression of the protein regulates expression of the EGF-R mRNA stability and protein levels in breast cancer cells. 3D-model analysis predicts a novel RNA-binding domain, which is the focus of ongoing structural studies. In summary, we have identified novel EGF-R mRNA-protein interactions that are the major determinant of EGF-R mRNA turnover in breast cancer cells. A family of novel bifunctional EGF-R mRNA-binding proteins has been identified that present new potential avenues for therapeutics based on selective targeting of the RNA-protein interaction.

## EXPRESSION OF THE NUCLEAR RECEPTOR COREPRESSORS, *NCOR* AND *SMRT*, IN OVARIAN TUMOURS.

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The stimulatory effects of steroid hormones, particularly estrogens, on normal breast and ovarian growth are well defined. In the ovary there is good evidence that estrogens synergise with follicle stimulating hormone (FSH) to stimulate granulosa cell proliferation. This effect of estrogen is mediated through estrogen receptor  $\beta$  (ER $\beta$ ), which is expressed in the normal FSH-stimulated granulosa cells; ER $\beta$  is also the predominant isoform expressed in granulosa cell tumours (GCT). Expression of the ER isoforms in ovarian tumours supports their role as important transcriptional activators of estrogen-regulated responses in ovarian tumourigenesis. The other important determinants of the ovarian response to estrogens are the coregulator (coactivator and corepressor) proteins. They have been implicated in the pathogenesis of breast and ovarian cancer through their association with nuclear receptors. We have previously examined the expression levels of the steroid receptor coactivators (SRC) in ovarian tumours; high levels of expression were observed in a panel of high-grade serous tumours. Several lines of evidence suggest that decreased levels of corepressor (NCoR and SMRT) expression may be responsible for rendering an antagonist inactive or even agonistic in advanced breast cancers. Therefore there is considerable interest in the possibility that the patterns of both coactivator and corepressor expression in hormone-dependent tumours may determine the nature of response to endocrine therapy. The aims of this study were to characterise the pattern of gene expression of NCoR and SMRT in a panel of ovarian tumours, and to contrast corepressor expression with that of coactivators. The patterns of gene expression were determined with total RNA from 8 premenopausal normal ovaries, 6 GCT and 15 epithelial tumours (serous and mucinous cystadenocarcinomas). RT-PCR assays were established for the corepressor genes, NCoR and SMRT, using gene-specific primers for NCoR (unpublished primer sequences provided by J. D. Graham, Denver, Colorado, USA). The SMRT primers span an exon-intron junction at which alternate splicing gives rise to 3 different isoforms.  $\beta_2$ -microglobulin ( $\beta_2$ -M) primers were included in a separate reaction as a control for RNA quality/quantity/loading. For a semiquantitative estimate of the relative levels of corepressor expression the amplicons from the PCR were subjected to Southern blot analysis using gene-specific internal probes. The 3 isoforms of SMRT were detected in the panel of ovarian samples: the ratio of the isoform expression levels are consistent between samples for each tumour type, except for the serous tumours where the ratio of the 3 isoforms varies between each tumour sample. Our initial studies reveal low levels of NCoR and SMRT expression in the serous tumours compared with the other tumour types and the normal ovaries. This is in marked contrast to coactivator expression which is highest in these tumours. Within each tumour type the levels of NCoR and SMRT vary more than we observed for the coactivators which suggests the corepressors may be important determinants of individual tumour behaviour. The biological role of the 3 isoforms of SMRT in these tumours, particularly the serous tumours is unknown. These preliminary results are consistent with increased coactivator levels and decreased corepressor levels having a role in tumour development and progression. It is tempting to speculate that the low levels of corepressor in these tumours may be responsible for the unrewarding response to estrogen/antiestrogen therapy seen for the ovarian tumours.

## KALLIKREIN 5/*KLK5* AND KALLIKREIN 7/*KLK7* ARE HIGHLY EXPRESSED IN EPITHELIAL OVARIAN CARCINOMAS

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The tissue kallikreins (KLKs) are a group of serine proteases that have been implicated in many disease processes including cancer. We have recently shown that kallikrein 4 (KLK4/hK4) is highly expressed in serous epithelial carcinomas compared to normal ovaries or other histological types. Previous studies have also reported that human kallikrein 5 (KLK5/hK5) and human kallikrein 7 (KLK7/hK7) are highly expressed in ovarian cancer. Thus, **the aims of this study** were further to examine the expression of KLK5/hK5 and KLK7/hK7 in normal ovaries and ovarian tumours with different histology, stage and grade in order to define the nature of KLK5 and KLK7 expression in ovarian cancer. Six Normal ovaries, 2 serous adenomas, 11 serous epithelial ovarian carcinomas, 8 serous epithelial-derived ovarian cancer cell lines, 4 mucinous epithelial tumours 5 endometrioid carcinomas, 3 clear cell carcinomas and 6 granulosa cell tumours were used in this study. Using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for 35 cycles and Southern blot analysis, we found that the KLK5 mRNA level is higher in serous epithelial-derived ovarian carcinomas and serous cancer cell lines (the majority of serous carcinomas examined were late stage disease) than normal ovaries, serous adenomas, endometrioid carcinomas and clear cell carcinomas, while no KLK5 expression was detected in mucinous tumours and granulosa cell tumours. KLK7 showed an essentially similar pattern as KLK5. Of interest, the serous carcinomas with high KLK5 mRNA levels showed high KLK7 expression as well. Northern blot hybridisation indicated an intense KLK5 and KLK7 transcript in serous ovarian carcinoma, whereas no visible band was detected in normal ovary. These results agree with and confirm the findings of RT-PCR and Southern blot analyses. In addition, the ovarian cancer cell line OVCAR-3 showed a larger KLK5 mRNA transcript than the human keratinocyte cell line, HaCat control. To determine the nature of the different KLK5 mRNA transcripts between OVCAR-3 and HaCat, 5'- Untranslated Region (5'UTR) sequence was examined. PCR primers were designed according to a human Expression Sequence Tag (EST) sequence from an ovarian carcinoma (Genebank number: BF03394). RT-PCR, semi-nested PCR and sequencing analyses were carried out and two novel sequences for an upstream exon of KLK5 were obtained in OVCAR-3, while another novel 5'UTR sequence was observed in normal ovarian epithelial cells. Both Western blot and immunohistochemistry analyses showed the higher expression of these two enzymes in ovarian carcinomas than normal ovaries and benign adenomas. In summary, our results demonstrate that, like KLK4, KLK5 and KLK7 are also highly expressed in serous epithelial ovarian carcinomas, particularly late stage disease. We have also shown that alternative transcripts of KLK5 are expressed in the ovary, although the significance of this finding is still to be determined. Our results further suggest that both KLK5 and KLK7 may be involved in ovarian tumour progression, as suggested for other KLK enzymes in other carcinomas.

## 8-CHLORO-CYCLIC AMP INDUCES APOPTOSIS AND G<sub>1</sub>-PHASE ARREST OF COV434 OVARIAN CANCER CELLS

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Ovarian granulosa cells (GCs) undergo dramatic proliferation during folliculogenesis. Proliferation of human GCs is controlled by multiple hormones and growth factors, including the gonadotrophins luteinising hormone (LH) and follicle stimulating hormone (FSH). Granulosa cell tumours (GCT) are the most frequent of all ovarian sex-cord stromal tumours, which comprise about 7% of all ovarian cancers. While little is known about the molecular genetic events involved in the aetiology of GCT, limited studies suggest that deregulation of gonadotrophin signalling to elements downstream of their receptors, such as the cAMP-regulated kinase PKA, plays a role in the biology of GCT. The aim of this study was therefore to evaluate the role of the gonadotrophin/cAMP/PKA pathway in the proliferation and survival of GCT cells. Using cultured COV434 cells as our model, treatment with the cell-permeable cyclic AMP analog 8-chloro-cyclic AMP inhibited the proliferation of COV434 cells as determined by growth assays and DNA flow cytometry, in a PKA-dependant manner. Growth inhibition was paralleled by a dramatic reduction in cyclin E- and D-dependant kinase (CDK) activities and decreased phosphorylation of their key substrate, the retinoblastoma protein. Inactivation of the major G<sub>1</sub> CDKs was accompanied by increased expression of the CDK inhibitors p21<sup>waf1</sup>, p16<sup>INK4A</sup> and p27<sup>kip1</sup> and enhanced association of these inhibitors with cyclin-CDK complexes. Furthermore, prolonged exposure to 8-Cl-cAMP resulted in the appearance of a significant proportion of cells with sub-diploid DNA content, indicative of apoptosis. These results demonstrate that the proliferation and survival of COV434 ovarian GCT cells is directly regulated by cAMP, thus providing a mechanism by which deregulation of gonadotrophin signalling may influence the aetiology of granulosa cell tumours.

## EFFECT OF INTRAVENOUS INFUSION OF LEPTIN ON THE PITUITARY-ADRENAL AXIS IN THE SHEEP FETUS.

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One of the major actions of leptin is to suppress neuropeptide-Y (NPY) secretion in the hypothalamus. Levels of leptin in blood are low in newborn infants of poor birth weight and abundance of leptin mRNA in fetal adipose tissue is positively correlated with fetal weight in sheep (Yuen, 1999). Since the hypothalamus of the sheep fetus expresses NPY (Warnes, 1998) it is possible that circulating leptin has similar actions in the fetus to those in adults. We hypothesise that endocrine actions of leptin in the fetus include suppression of hypothalamic NPY and, consequently, reduced secretion of AVP and CRH, leading to reduced circulating ACTH and glucocorticoids. Leptin therefore could promote fetal growth, by indirect suppression of adrenal glucocorticoid synthesis. Cortisol in the sheep fetus initiates parturition. We therefore measured the effect of i.v. infusion of leptin into fetal sheep on the timing of labour and on the concentrations in fetal blood of ACTH and cortisol. Under general anaesthesia between 115 and 121 d of pregnancy (term = 150 +/- 3 d), ewes and fetuses were chronically cannulated. At 144 d gestation, fetuses were randomly assigned to receive either ovine leptin at 1.06 +/- 0.02 mg/kg per d (n=5) or saline (n=4) until periodic contractions were detected by measurement of amniotic pressure. ACTH and cortisol were measured in fetal plasma collected in the 24 h preceding labour. I.v. infusion of leptin into fetal sheep did not effect the timing of labour (150 +/- 1 d gestation). Similarly, the concentrations in fetal plasma of neither ACTH (165 +/- 43 ng/L) nor cortisol (281 +/- 37 nmol/L) were affected by leptin treatment. This shows that intravenous infusion of leptin does not suppress the pituitary-adrenal axis in fetal sheep in the last days before labour. Therefore, if leptin promotes fetal growth, it is unlikely to do this through actions on the pituitary-adrenal axis.

## PLASMA LEPTIN CONCENTRATIONS IN SHEEP FOLLOWING DIETARY RESTRICTION

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Leptin a hormonal product of the *ob* gene is secreted primarily by fat cells, and was first described as a regulator of adiposity, food intake and energy metabolism (1). Although it has been shown that plasma leptin levels are highly correlated with body fat in humans and rodents, the mechanisms which modulate leptin levels remain unclear. From these studies it could be concluded that leptin is an important humoral signal to the central nervous system on body composition and regulation of food consumption. We have shown that plasma leptin levels in sheep, in contrast to rodents, are not responsive to short term changes in blood glucose or insulin, as has been shown in humans (2).

In this study the effect of dietary restriction on serum leptin was compared between a group of Coopworth sheep selected for a greater subcutaneous fat body composition (3) and a group of normal controls. The two groups (including both sexes) were divided randomly into 2 treatment groups and assigned to the following diets: 60% and 100% of the maintenance diet. They were fed for 6 weeks at a level to maintain their body weight and then the restricted groups had their diet reduced for 12 weeks followed by restoration to the maintenance diet for another 12 weeks. Blood samples were taken weekly and the animals scanned at 6, 21 and 27 weeks into the experiment using a whole body X-ray computer aided tomography system (Hitachi CTW-430). Leptin was measured as previously described (2).

All sheep on the 60% diet significantly ( $P < 0.05$ ) lost weight and all failed to fully regain their original weight when returned to the maintenance diet. The relative amount of weight lost was similar between the normal and fat groups with approximately 10-12% loss in weight in the 60% group with the control groups losing no more than 2%. In the normal group 56% of the weight lost was fat, while in the fat group only 38% of the weight lost was fat. In the fat group leptin declined significantly ( $P < 0.05$ ) with the onset of the dietary restriction which returned to normal within 2 weeks when the maintenance diet was restored. In the 60% normal group there no significant ( $P > 0.05$ ) change in leptin observed, and in the non-restricted groups there was no change in plasma leptin concentrations. These data indicate that in normal individuals leptin is not involved in modulating body composition, but that there is an uncoupling of the leptin regulatory mechanisms in sheep that tend to conserve fat. These preliminary data are extremely interesting and suggest that this sheep model may prove useful in investigating the role of leptin in obesity.

## MODULATORY EFFECT OF RECOMBINANT OVINE LEPTIN ON GH, GHRH RECEPTOR AND GHRP RECEPTOR SYNTHESIS IN PRIMARY CULTURED OVINE SOMATOTROPES

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Leptin is a peptidergic hormone secreted from adipose tissue to reduce food intake and increase energy expenditure. Growth hormone (GH) from pituitary gland increases protein synthesis and reduces formation and proliferation of fat cells. Although existing data suggest an influence of leptin on circulating levels of GH, reports on the acting site and properties of leptin action on GH levels are still controversy. Species difference of the effect of leptin on GH secretion was also noted between rats and sheep or humans. Present study was designed to investigate the direct action of ovine leptin on ovine somatotropes *in vitro* by analysing the expression levels of GH, GH-releasing hormone receptor (GHRH-R), and GH-releasing peptide receptor (GHRP-R). In primary cultured ovine somatotropes (60-80% of total cells were somatotropes), treatment of cells with recombinant ovine leptin (*ro*-leptin, 10 nM) for 3 days with fresh replenishment of *ro*-leptin once per day significantly reduced GH secretion in response to GHRH (10 nM). GHRP-2 (100 nM)-stimulated GH secretion was however increased by the same treatment with *ro*-leptin. The combined effect of GHRH and GHRP-2 on GH secretion was not altered by the treatment of cells with *ro*-leptin. Intracellular mRNA levels coding for ovine GH, GHRH-R and GHRP-R were analysed by semi-quantitative RT-PCR methods. GH and GHRH-R mRNA levels in cultured somatotropes were decreased by the treatment of cells with *ro*-leptin (1-100 nM) for 1 to 3 days in a dose- and time-dependent manner. One-day treatment of cells with high dose (100 nM) of leptin reduced both GHRH-R and GH mRNA levels but three-day treatment with as low as 1 nM leptin decreased both mRNA levels. Levels of GHRP-R mRNA were, however, increased by three-day treatment of cells with *ro*-leptin (1-100 nM). One- or two-day treatment of cells with *ro*-leptin (up to 100 nM) did not significantly alter the levels of mRNA coding for GHRP-R. These results suggest that leptin has a long-term effect on ovine somatotropes to reduce GH and GHRH receptor synthesis leading to a decrease in GHRH-stimulated GH secretion. Leptin appears, however, to have an opposite effect on GHRP receptor synthesis by increasing the expression of GHRP-R, which leads to an increase in GHRP-stimulated GH secretion. Combined effect of GHRH and GHRP-2 seems to be maintained after *in vitro* treatment of cells with *ro*-leptin, which suggests a possible therapeutic use of GHRP in the treatment of GH deficiency in obesity patients. Supported by Australian NHMRC and Aza Research Pty Ltd.



## LEPTIN RECEPTOR EXPRESSION IN RAT PLACENTA: CHANGES OVER LATE GESTATION AND SUPPRESSION BY GLUCOCORTICOIDS

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Leptin, the peptide hormone product of the *ob* gene, reaches maximal levels in maternal and fetal plasma approaching term and may exert a positive effect on fetoplacental growth. Leptin actions are mediated via a 120 kDa receptor, and a shorter isoform of this receptor is believed to act as a transporter of leptin through physiological barriers. Hence, placental expression of leptin receptor may act as a local mediator of leptin action or as a transporter of maternal leptin to the fetus, and both may be important in relation to fetoplacental growth. In the present study, therefore, leptin receptor protein expression was examined in the rat placenta during the latter stages of pregnancy. In addition, because glucocorticoids inhibit fetoplacental growth, the effect of excess glucocorticoid exposure on leptin receptor was also investigated.

Placentas were obtained from rats at gestational ages 16 and 22 days (g16 and g22), covering the period of maximal fetal growth (term = day 23). Glucocorticoid exposure was increased directly by administration of dexamethasone acetate (DEX) or indirectly by carbenoxolone (CBX, an inhibitor of 11 $\beta$ -HSD). Western blot analyses were performed on the two morphologically- and functionally-distinct regions of the rat placenta, the basal and labyrinth zones, using a primary antibody against the common region of the leptin receptor (Santa Cruz, sc#1835). Immunocytochemical analyses were also performed using this antibody.

Immunoreactive leptin receptor signals (50 and 120 kDa) were evident in the basal and labyrinth zones of the placenta at both stages of pregnancy. Expression of the 120 kDa species fell significantly in the basal zone between g16 and g22, but the 50 kDa species remained unchanged. Interestingly, immunocytochemical localisation of the leptin receptor occurred within fetal blood vessels of the placental labyrinth zone on both days. Both DEX and CBX reduced ( $P < 0.05$ ) expression of the 120 kDa species in the basal zone at g22 (47% and 28% lower, respectively). These treatments had no effect on expression of the 120 kDa species in the labyrinth zone or the 50 kDa species in either placental zone.

These data demonstrate that the expression of the 120 kDa leptin receptor decreases in the basal zone of placenta during late pregnancy and after increased glucocorticoid exposure. These changes are likely to inhibit the responsiveness of the placenta to leptin and thus provide a possible mechanism via which glucocorticoids retard placental growth.

## THE EXPRESSION OF THE LONG (OB-RB) AND SHORT (OB-RA) LEPTIN RECEPTORS ACROSS THE ESTRUS CYCLE IN THE MATURE RAT OVARY.

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Leptin is secreted by adipocytes and exerts its effects by interacting with the long form of the leptin receptor, OB-RB. The leptin protein and leptin receptors have been localized in the ovary and acute leptin treatment directly inhibits ovulation in the rat ovary. We hypothesized that leptin receptor (OB-R) gene expression may vary throughout the estrus cycle in order to modulate the sensitivity of the ovary to leptin. In this study, gene expression of OB-RB and OB-RA in the adult ovary was investigated at different stages of the rat estrus cycle, determined by vaginal cytology. Ovaries were collected and RNA extracted for real time RT-PCR of OB-R gene expression. OB-RB gene expression (fg RNA/ $\mu$ g genomic DNA) was lower in the proestrous ( $3.13 \pm 0.18$ ) and diestrous ( $2.52 \pm 0.19$ ) stages of the estrus cycle while there were higher levels of expression in metestrous I ( $5.9 \pm 0.27$ ) and II ( $4.6 \pm 0.24$ ) stages ( $p < 0.001$ ). Expression (fg RNA/ $\mu$ g genomic DNA) of OB-RA was at its maximum in the metestrous II ( $0.0655 \pm 0.0008$ ) stage, at high levels in the metestrous I ( $0.039 \pm 0.0008$ ) stage and the lowest levels were found at proestrous ( $0.005 \pm 0.0002$ ) and diestrous ( $0.0011 \pm 0.00009$ ) stages ( $p < 0.001$ ). Plasma estradiol levels (pmol/L) were highest at proestrous ( $71.7 \pm 4.8$ ), and similar between metestrous I ( $50.8 \pm 6.9$ ), metestrous II ( $46.0 \pm 3.6$ ) and diestrous ( $38.1 \pm 3.2$ ) ( $p < 0.05$ ). Plasma progesterone levels (nmol/L) were higher in the luteal phases of the cycle (metestrous II:  $59.2 \pm 7.2$ , diestrous:  $46.6 \pm 8.1$ ) than proestrous ( $16.3 \pm 2$ ) and metestrous I ( $19.0 \pm 2.7$ ) ( $p < 0.05$ ). Plasma leptin levels were only detectable in proestrous ( $0.35 \pm 0.05$  ng/ml) and were below the detection limit of the assay at other stages of the estrus cycle. In summary, OB-RB and OB-RA are lower at proestrous and diestrous than the metestrous stages of the rat estrus cycle. The fluctuations in OB-R may be a response to the levels of circulating steroid hormones and leptin. This research supports our initial hypothesis and shows that ovarian leptin receptor concentrations vary throughout the estrus cycle in response to the changing environment of the ovary.

## LEPTIN RECEPTOR EXPRESSION IN THE ENDOMETRIUM AND MYOMETRIUM OF THE RAT

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Leptin, the product of the *ob* gene, influences body weight via regulation of food intake and metabolic rate. In addition, leptin is thought to impact on the function of several reproductive tissues including the ovary, placenta and uterus. Because leptin exerts angiogenic effects in some models, it has been proposed that leptin may play an important role in implantation and placentation. The leptin receptor (Ob-R) has been detected in the rat uterus but its precise localisation and regulation are unknown. In the present study, therefore, the immunohistochemical localisation of Ob-R was examined in the rat uterus throughout the estrous cycle and following ovariectomy with or without oestrogen replacement.

Uteri were collected from Wistar rats at each stage of the 4-day oestrous cycle (proestrus, oestrus, post-oestrus and dioestrus) as determined by daily vaginal smears. Uteri were also collected from two additional groups of rats 10 days after bilateral ovariectomy (OVX). One of these groups was treated with oestradiol delivered via a mini-osmotic pump at a dose of 40 ng/h for the three days prior to collection.

In cycling rats, Ob-R was immunolocalised to both the glandular and luminal epithelium of the endometrium and in the myometrium at each cycle stage. Staining intensity appeared greatest at proestrus and oestrus, those cycle stages when oestrogen levels are maximal. Western blot analysis of whole uteri confirmed the presence of a 50 kDa and a 120 kDa species of Ob-R in the cycling rats. Similarly, Western analysis of whole uteri from OVX rats showed expression of these two immunoreactive species, and both appeared to increase following oestrogen replacement.

In conclusion, this study shows that the Ob-R is expressed in both the endometrium and myometrium of the rat uterus, and that this expression appears to vary with oestrogen. Moreover, two distinct immunoreactive species are expressed in the uterus. These observations indicate that leptin could play an important role in mediating oestrogen-related changes in uterine function, possibly in relation to implantation.

## EXOGENOUS GONADOTROPHIN CONTROL OF INHIBIN SUBUNITS, ACTR-1 AND SMAD2 DISTRIBUTION IN HPG MOUSE OVARY

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**Aims:** To investigate the patterns of expression of inhibin  $\alpha$ -subunit,  $\beta$ A and  $\beta$ B subunits, activin receptor-1 and Smad2 by immunohistochemistry in *hpg* mice (gonadotropin deficiency) after treatment with hrecFSH/hrecCG alone or in combination

**Methods:** Homozygous *hpg* female mice (21-23 days of age) were studied following: no treatment; recombinant hFSH alone (10 IU/day) for 20 days; simultaneous hFSH (10 IU/day) + recombinant hCG (1IU/day) for 20 days; hCG alone (1 or 10 IU/day) for 20 days; sequential hFSH (10 IU/day) for 13 days then hCG (10IU/day) for 7 days; hFSH for 20days with a single dose of hCG. Mice were killed by cardiac exsanguination, ovaries were fixed in 4% paraformaldehyde in PBS (pH7.2-7.4) for 24 hours, processed, embedded in paraffin, and sectioned at 6 $\mu$ m thickness. Immunohistochemistry was performed with the following primary antibodies: inhibin  $\alpha$ -subunit (goat polyclonal IgG, Labsupply Australia), inhibin  $\beta$ A and  $\beta$ B subunits (mouse anti human E4 and C5 provided by Prof. Groome, Oxford, UK), activin receptor-1 (rat polyclonal IgG provided by Dr Kenji Okazaki, Japan) and smad2 (rabbit polyclonal IgG provided from Ludwig Institute for Cancer Research, Japan). Immunostaining for inhibin  $\beta$ A and  $\beta$ B subunits was obtained by using a biotinylated primary antibody to identify specific binding of the mouse monoclonal antibodies used.

**Results:** FSH alone stimulated follicle development to preovulatory stage. No corpora lutea were seen unless hCG was co-administered. hCG alone caused theca cell proliferation with little growth of follicles. Inhibin alpha subunit immunostaining was seen in the granulosa cells of healthy follicles only and not in atretic follicles, theca cells, oocyte or corpora lutea. Chronic treatment with hFSH/hCG had no effect on localisation. Inhibin betaA and Inhibin betaB immunostaining was seen in granulosa cells, oocytes of developing follicles, theca cells of normal bigger follicles and corpus luteum but not seen in theca cells of primary/secondary or in atretic follicles. Treatment with FSH increased immunostaining in granulosa cells and oocytes while addition of hCG led to immunostaining in theca cells round early follicles. Smad2 and ActRIB showed similar immunostaining distribution to beta-subunits, in oocytes, theca cell, of developing follicles and corpora lutea. Increased staining was seen in larger follicles with relatively little in smaller follicles, an effect that was increased by gonadotrophin treatment. Administration of a single dose of hCG after hFSH produced a dramatically different picture with inhibin immunostaining with absent alpha subunit immunostaining in the large antral follicles.

**Conclusions:** During chronic gonadotrophin treatment, hCG has a greater stimulatory effect on localisation of inhibin beta A and B subunits, ActR1 and Smad2 than FSH, while no change of alpha subunit distribution was observed. The immunolocalisation of beta-A and B distribution may represent bound ligand as well as site of synthesis. The acute effects of a single dose of hCG suggest that hCG/LH is involved in down-regulation of inhibin alpha subunit synthesis. This effect is consistent with a role for inhibin subunits in ovulation.

## STUDY BY *IN SITU* HYBRIDISATION OF EXPRESSION OF INHIBIN AND ACTIVIN RECEPTORS IN MOUSE OVARY.

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**Aim:**

Activin and inhibin are dimeric proteins with paracrine actions within the oocyte. Activin exerts its physiological action by binding to type II receptor kinase (ActRII and IIB) to promote the phosphorylation of type I receptor kinase (ALK4). Inhibin has been reported to exert antagonistic effects through binding to betaglycan, a type III TGF-beta receptor that has been reported to function as an inhibin co-receptor with ActRIIB. As a prelude to detailed study of the gonadotrophic control of inhibin/activin paracrine actions, preliminary studies investigated ActRIIB and betaglycan expression in mouse ovary with an oocyte-specific protein (GDF-9) studied for comparison.

**Method:**

6 week olds mouse ovary was used for in situ hybridization. Primers for mouse ActRIIB (341bp), mouse betaglycan (434bp) and mouse GDF-9 (467bp) were designed according to specific gene sequences in GeneBank. PCR products for these genes were cloned to pCRII-TOPO vector (Invitrogen). A single strand RNA probe was generated using RNA polymerase T7 and SP6. Antisense and sense probes were labelled with digoxigenin-UTP (Roche). A non-radioactive in situ hybridization method was used to localize mRNA of ActRIIB, betaglycan and GDF-9. Hybridization of antisense probes to complementary mRNA was detected by a chromogenic staining reaction catalyzed by an anti-digoxigenin antibody-alkaline phosphatase conjugate. The slides were washed in (0.1x SSC) high stringency buffer and RNase A also used in this method.

**Result:**

1) ActRIIB mRNA was mainly localized in granulosa cells at different stages of follicular development including primordial follicles. ActRIIB was also detected in thecal cells in corpus luteum and oocyte, the expression levels of ActRIIB were lower compared to granulosa cells and thecal cells. No ActRIIB expression was detected in interstitial cells.

2) With betaglycan, the majority of mRNA was localized in granulosa cells at different stages with the same pattern as ActRIIB. A lower level of betaglycan mRNA was observed in the oocyte. No betaglycan mRNA was detected in interstitial cells.

3) The expression of GDF-9 was detected in the oocyte only. No GDF-9 was detected in granulosa cells, thecal cells and interstitial cells.

**Conclusion:**

The principal site of action for both inhibin and activin is likely to be the granulosa cells (including corpus luteum) with less intense receptor localisation in the oocyte and thecal cells.

## EXPRESSION OF ACTIVIN RECEPTORS BY HUMAN ENDOMETRIAL STROMAL CELLS: EVIDENCE FOR A ROLE FOR ACTIVIN A IN FACILITATING DECIDUALISATION

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Endometrial stromal cells undergo spontaneous decidualisation in the mid-late secretory phase of the menstrual cycle, involving dramatic changes in cell morphology and function, to provide an environment suitable for embryo implantation. This differentiation is induced by progesterone, and involves numerous paracrine factors including PGE, IL-11, and cAMP. Activin BA and BB subunits are expressed by decidualising stromal cells<sup>1</sup>. Many known actions of activin A on cell differentiation and during tissue remodelling would be consistent with a role in promoting decidualisation. Activin signals through interaction with serine / threonine kinase activin receptors (ActR), which are members of the TGF-β receptor superfamily. Activin type II receptors are required for ligand binding, which stimulates type I receptor recruitment and activates signal transduction through phosphorylation of intracellular Smads. To determine the site of action of activin in the endometrium, ActRIa, Ib, IIa and IIb subtypes were localised by immunohistochemistry. All receptor subtypes were localised exclusively to stromal cells, including decidualised stromal cells. This localisation was confirmed by in situ hybridisation for all ActR mRNA transcripts. Fluctuations in receptor subtype mRNA expression across the menstrual cycle and in early pregnancy was assessed by semi-quantitative and real time RT-PCR. Amplicons of expected lengths were detected in positive control tissues (term placenta and HepG2 hepatoma cell line) and were sequenced to confirm identity. ActRIa, ActRIIa and ActRIIb mRNA expression was detectable in endometrium throughout the menstrual cycle, with maximal expression in the early and mid secretory phases and in early pregnancy decidua. ActRIb mRNA expression was only detectable in endometrial samples during the early and mid secretory phase and early pregnancy. Activin receptors are therefore expressed by stromal cells with maximal mRNA expression in the early-mid secretory phase, appropriate for a role for activin stromal cell decidualisation. To further explore the functional significance of activin in the endometrium, stromal cells isolated from endometrial biopsies were treated with 17β-estradiol (E<sub>2</sub>) and medroxy-progesterone-acetate (MPA) to induce decidualisation *in vitro*, in the presence or absence of activin A (1-100ng/ml). Production of prolactin (an established marker of stromal cell decidualisation) was measured by ELISA in conditioned medium. Prolactin was detectable after 6-8 days of treatment when cells were treated with E<sub>2</sub> + MPA alone. Activin A increased prolactin production in a dose dependant manner, with a 7.5 fold increase with 100ng/ml, compared to E<sub>2</sub> + MPA alone. In summary, activin receptors are expressed by endometrial stromal cells, with elevated mRNA expression immediately prior to and during the induction of decidualisation. High expression levels were also present in decidua during early pregnancy, correlating with more extensive decidualisation. This spatial and temporal expression is consistent with a role for activin in facilitating stromal cell decidualisation. Furthermore, preliminary functional studies indicate that activin A enhances decidualisation *in vitro*.

## TGF $\beta$ DOES NOT ANTAGONISE INHIBIN ACTIONS IN A GONADOTROPE CELL LINE (L $\beta$ T2) EXPRESSING BETAGLYCAN AND P120.

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Activin stimulates expression of gonadotropin releasing hormone receptor (GnRHR) and follicle stimulating hormone (FSH)  $\beta$ -subunit in gonadotropes. Inhibin antagonizes activin actions on the gonadotropes, but its molecular mechanism of action remains poorly understood. It has been suggested that inhibin exerts its antagonistic effects by competing with activin for the binding of the activin receptor complex. Betaglycan, a type III TGF $\beta$  receptor, and InhBP have recently been identified as inhibin-binding accessory proteins in this process. To evaluate the effect of inhibin on activin-induced transcription in gonadotrope cells we used two luciferase reporter constructs, the first containing 5.5 kb of the ovine FSH $\beta$  promoter (oFSH $\beta$ luc) and the second containing three copies of the activin-responsive sequence of the GnRHR promoter (3XGRAS-PRL-lux). These constructs were transfected into the mouse L $\beta$ T2 cell line which is capable of producing FSH. The addition of activin-A (0 - 5 nM) resulted in a dose-dependent increase of oFSH $\beta$ luc activity by up to 2-fold. Inhibin-A alone (0 - 1 nM) did not significantly alter oFSH $\beta$ luc activity. However, the luciferase activity stimulated by 0.5 nM activin-A was decreased by up to 50% in a dose-dependent manner by inhibin-A or inhibin-B, with the latter being 10 times less potent than inhibin-A. Activin-A (0.5 nM) induced a 10-fold increase of 3XGRAS-PRL-lux activity, while 0.5 nM inhibin-A reduced the activin-stimulated activity by more than 50%. TGF- $\beta$ <sub>1</sub> (0 - 4 nM) alone did not significantly stimulate either the oFSH $\beta$  promoter or the GnRH receptor promoter element in L $\beta$ T2 cells. This contrasts with other activin-responsive cells and promoters which normally also respond to TGF- $\beta$ . Since both inhibin and TGF- $\beta$  bind betaglycan, we examined whether TGF- $\beta$  can modulate inhibin bioactivity through competition for betaglycan binding. We determined by RT-PCR the presence of betaglycan mRNA in L $\beta$ T2 cells, consistent with the potent activity of inhibin observed on these cells. TGF $\beta$ <sub>1</sub> (IC<sub>50</sub> = 900 pM) competed with [<sup>125</sup>I]inhibin for binding to L $\beta$ T2 cells, and reduced the affinity labelling of betaglycan on the surface of the cells. However, co-incubation of 0.5 nM inhibin-A with TGF- $\beta$ <sub>1</sub>, up to 2 nM, did not result in restoration of the activin-induced FSH $\beta$  promoter activity. These results suggest that TGF- $\beta$ <sub>1</sub> competition with inhibin for binding to betaglycan does not interfere with inhibin's suppression of activin-induced FSH and GnRHR production in the gonadotrope cell line L $\beta$ T2. *Supported by the NHMRC of Australia (RegKey 983212) and by FCAR funds, Canada.*

## RELEASE OF ACTIVIN A DURING SYSTEMIC INFLAMMATION IS BIPHASIC, WITH THE INITIAL SECRETION PRECEDING THAT OF TUMOUR NECROSIS FACTOR- $\alpha$ .

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Activin A was first isolated as a regulator of FSH, but more recently roles for this factor have emerged in many non-reproductive systems, including that of an inflammatory cytokine. Current evidence suggests that activin A is stimulated by proinflammatory cytokines in responsive cell types and that activin may play a key role in inflammation via its ability to antagonise interleukin (IL)-1 and IL-6 signalling. Lending credence to the concept of activin A as an inflammatory regulator, we have documented that circulating activin A concentrations are increased very early in systemic inflammatory episodes (1). The timeframe of release in relationship to key pro-inflammatory cytokines, such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), is presently ambiguous, so the aim of this study was to carefully dissect this early increase in activin A using a more rigorous sampling regime. To induce systemic inflammation, an intravenous injection of lipopolysaccharide (LPS, 50 $\mu$ g, n=5) or non-pyrogenic saline (n=3) was given to Corriedale ewes. Blood was collected via indwelling jugular catheters every 10 minutes in the first hour, then every 15 minutes out to 8 hours. Plasma samples were measured for TNF- $\alpha$ , IL-6 and total activin A and follistatin, using specific immunoassays, and rectal temperatures recorded using a digital thermometer. All data were analysed using repeated measures analysis of variance, with a Dunnett's post-hoc test. Following LPS injection, fever was induced in a biphasic profile with peaks at 75 minutes and 180 minutes. TNF- $\alpha$  increased initially 40 minutes after injection and peaked at 75 minutes, with levels returning to control values within 6 hours of LPS. IL-6 displayed a more delayed response with significant elevation by 4 hours and remaining elevated for at least eight hours. Surprisingly, activin A concentrations displayed a biphasic profile in response to an injection of LPS and followed a profile very similar to that seen with the fever response. The initial rise in activin A was noted earlier than for TNF- $\alpha$  (30 vs 40 minutes), with peaks occurring at 50 and 200 minutes, and a return to baseline values by 6 hours after LPS. These observations provide new information that activin A is an early marker of systemic inflammation and has relevance to clinical conditions such as septicaemia and meningitis. The biphasic release of activin suggests that there may be two distinct mechanisms and/or sites of release, which we are currently investigating. *Supported by the NHMRC of Australia (Program Grant 973218).*

## A NATURALLY-OCCURRING MUTATION OF THE INHIBIN $\alpha$ -SUBUNIT GENE ASSOCIATED WITH PREMATURE OVARIAN FAILURE DOES NOT ALTER THE FSH-SUPPRESSING ACTIVITY OF INHIBIN A *IN VITRO*

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Premature ovarian failure (POF), a condition associated with the cessation of ovarian function under the age of 40, is characterised by amenorrhoea, low estrogen and elevated serum gonadotropins levels. POF is a highly heterogeneous condition, and though various sex chromosome abnormalities and autoimmune disorders are contributing factors, its etiology is generally unknown. Recently, Shelling *et al* (Hum Reprod 2000, 15:2644) reported an association between a naturally-occurring mutation in the inhibin  $\alpha$ -subunit gene and POF. This single nucleotide mutation results in a change of residue 257Ala to Thr. Inhibin plays an important role in the negative feedback control of FSH secretion. In women, FSH regulates the selection and development of ovarian follicles. A loss of inhibin bioactivity resulting from this mutation may therefore lead to excess FSH secretion and the subsequent premature depletion of follicles. Therefore, to assess whether this mutation affects inhibin bioactivity, we produced recombinant wild type and mutant human inhibin A and determined the ability of each to inhibit FSH secretion in cultured rat pituitary cells. COS-7 cells were transiently transfected with two expression plasmids, one containing either wild type or mutated inhibin  $\alpha$ -subunit sequences, and one containing  $\beta$ A-subunit sequence. Secreted inhibin proteins were isolated by immunoaffinity chromatography using a monoclonal  $\alpha$ -subunit antibody. Further purification by reverse-phase HPLC gave 2 inhibin-containing peaks when assayed by the  $\alpha$ - $\beta$ A subunit ELISA. Western blot analysis using either  $\alpha$ - or  $\beta$ A-subunit-specific antisera indicated that the major peak (peak I) contains predominantly mature 34 kDa inhibin A with small amounts of a 39 kDa glycosylation variant and a 97 kDa band consistent with the  $\alpha$ N- $\alpha$ C/Pro- $\beta$ A, 116 kDa) form of inhibin. On reduction, these three bands resolved into two separate  $\alpha$ -subunit bands of 24 kDa and 31 kDa, consistent with the differential glycosylation of mature  $\alpha$ C region. Peak II comprised predominantly of higher molecular mass (60-116 kDa) inhibin species detectable by both  $\alpha$ - and  $\beta$ -subunit antisera and were identified as full length (Pro- $\alpha$ N- $\alpha$ C/Pro- $\beta$ A, 116 kDa) and glycosylation variants of Pro- $\alpha$ N- $\alpha$ C/□□ (60-68 kDa). The FSH-suppression activities of inhibin A in both wild type and mutant peak I and II were measured in a rat pituitary cell bioassay (B), and compared with their  $\alpha$ - $\beta$ A immunoactivities (I) deduced from the ELISA measurements. The B/I ratios (mean  $\pm$  SD, n=3) for wild-type and mutant peak I materials were  $0.95 \pm 0.35$  and  $1.07 \pm 0.35$  respectively. Similarly, peak II gave B/I ratios of  $1.37 \pm 0.34$  and  $1.81 \pm 0.42$  respectively, indicating that there are no differences in the FSH-suppressing activities between wild-type and mutant inhibin A. We conclude that the  $\alpha$ -subunit Ala257Thr mutation does not result in decreased inhibin A bioactivity, as measured by its ability to inhibit FSH secretion *in vitro*. However, since inhibin occurs as two common isoforms, inhibin -A and -B, we are currently assessing whether the mutation affects inhibin B bioactivity.

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## ONE HEALTHY BABY AS THE ENDPOINT OF OVULATION INDUCTION

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Authorities have recently drawn attention to the marked increase in the number of twins, triplets and higher order multiple pregnancies in the USA following ovulation induction in a program of "controlled" ovarian hyperstimulation – intrauterine insemination (COH-IUI) (1,2,3). There has been particular obstetric concern because even twin pregnancies are known to have increased risks of perinatal mortality and cerebral palsy. Our aim was to review our COH-IUI Program in a retrospective consecutive series between 1997-1999. At the Monash Ovulation Induction Service, 178 women underwent 378 cycles of COH-IUI. Patients were stimulated with recombinant human FSH (rhFSH, median 112IU; range 75-150). Monitoring was undertaken with transvaginal ovarian ultrasound and twice weekly serum estradiol assays. Administration of human chorionic gonadotropin (HCG) was given when no more than 3 follicles were  $\geq 14$ mm diameter. IUI was performed 36 hours after HCG. This was a more conservative approach than previously reported by others (2,3) and was used in an attempt to minimise multiple pregnancy rates. There was a total of 33 pregnancies. 21 conceptions were singleton, 5 were twins and there were 2 triplet conceptions. In addition, 5 pregnancies ended in early miscarriage prior to vaginal ultrasound assessment. The incidence of multiple pregnancy was 21%. The conception rate was 8.7% per cycle and 18% per patient commenced on treatment. 28 women gave birth: there were 22 singleton births, 5 twins and 1 triplet birth. The livebirth rate was 7% per cycle and 15.7% per patient. With the above guidelines and monitoring, 22/28 births (78%) in this COH-IUI program were of a normal singleton baby at term gestation.

## BODY COMPOSITION AND ENERGY METABOLISM IN NORMOTENSIVE AND HYPERTENSIVE PREGNANCY.

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Hypertension and obesity are insulin resistant states in non-pregnant adults. Pregnancy is an insulin resistant state, however it is not known whether hypertensive pregnancy is associated with greater insulin resistance or increased fat mass. The aim of this study was to determine whether the insulin resistance syndrome and altered body composition were features of hypertensive pregnancy. A cross-sectional study was performed. Women were recruited in their 3<sup>rd</sup> trimester of pregnancy from the antenatal clinic, day assessment unit, and maternity ward of St George Hospital. Women with preeclampsia PE (n=12), gestational hypertension GH (n=12), essential hypertension in pregnancy EH (n=11), and normotensive pregnancy NP (n=10) were recruited. Energy metabolism was assessed by indirect calorimetry to measure basal metabolic rate (BMR) and diet-induced thermogenesis (DIT). Body composition was measured as lean body mass, total body water (TBW) and fat mass by bio-electrical impedance. Blood was collected for measurement of glucose, insulin and lipid profiles. Insulin resistance was indirectly assessed by the insulin and glucose concentrations and DIT. (\* p < 0.05 vs NP)

	weight	Lean mass	Fat mass	TBW	BMR	DIT
Normal P	80.8 ± 2.5 kg	50.4 ± 0.9 kg	30.4 ± 2.1 kg	35.2 ± 0.6 L	1668±65 kcal/24h	2006±64 kcal/24h
EH	88.9 ± 5.4 kg	50.7 ± 2.7 kg	37.1 ± 3.5 kg	38.3 ± 1.7 kg	1494±117 kcal/24 h*	1857±113 kcal/24 h
GH	90.7 ± 4.0 kg *	53.8 ± 1.8 kg	37.0 ± 2.6 kg	38.5 ± 1.3 kg	1677±95 kcal/24 h	1872±91 kcal/24 h *
PE	81.5 ± 4.3 kg	49.3 ± 1.9 kg	32.2 ± 2.6 kg	35.7 ± 1.5 L	1467±55 kcal/24 h	1672±81 kcal/24h

All groups were not significantly different in age, height or weeks of gestation. Women with EH (77 ± 5.0 kg) and GH (76.1 ± 3.7 kg) were heavier than NP (64.8 ± 2.4 kg, p < 0.05) women pre-pregnancy. In the 3<sup>rd</sup> trimester, women with GH (p < 0.05) were heavier than NP whereas PE were similar to NP. No significant differences in lean body mass, fat mass and total body water were detected between the groups. EH women were otherwise similar to NP but GH women had a reduced DIT (p < 0.05) and almost double insulin levels (p < 0.05). PE women had a similar body composition and insulin levels but reduced BMR, DIT and glucose levels compared to NP. Women who develop GH, but not PE, are more likely to be overweight. EH women are similar to NP women throughout pregnancy. Both GH and PE appear to be associated with some degree of insulin resistance, greater than that occurring in normal pregnancy.

## TRANSDERMAL TESTOSTERONE THERAPY IMPROVES WELL-BEING, MOOD AND SEXUAL FUNCTION IN PREMENOPAUSAL WOMEN.

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**Background:** Our understanding of the actions of testosterone in women is incomplete with no consensus as to what constitutes either biochemical or clinical testosterone deficiency. The focus of the limited research into testosterone replacement has been on sexuality in postmenopausal women with little attention to those in their reproductive years. However, the influence of testosterone on well being and mood also requires further exploration.

**Methods:** Premenopausal women with low libido and low total serum testosterone participated a randomized placebo-controlled cross-over study of testosterone cream (10mg/day) with two 12 week treatment periods separated by a 4 week wash out period.

**Results:** Thirty-four women completed the study per protocol, with 31 women (mean age: 39.7 ± 4.2 years, total testosterone 1.07nmol/L ± 0.50) providing complete data. Testosterone therapy resulted in clinically relevant and statistically significant improvements in the composite scores of the Psychological General Well-Being Index (p=0.003) and the Sabbatsberg Sexual Self-rating Scale (p=0.001) compared to placebo. A clinically significant decrease in the Beck Depression Inventory score approached significance (p=0.06). Mean total and bioavailable testosterone levels during treatment were in the high normal range and estradiol was unchanged. No adverse effects were reported.

**Conclusions:** Testosterone therapy improves well-being, mood and sexual function in premenopausal women with low libido and low testosterone, providing strong evidence for a female androgen deficiency syndrome.

## LONG-TERM OUTCOMES OF ELECTIVE HUMAN SPERM CRYOSTORAGE

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Elective sperm cryostorage is an established technique for men about to undergo treatment likely to render them subfertile or sterile. However, such fertility insurance is costly and long-term outcomes have not been widely reported. We reviewed the outcomes of semen samples stored from 930 men over a 22 year period in a single urban academic teaching hospital. Among these men, 1.4% were too ill to provide semen and 9.0% had samples unsuitable for cryostorage due to azoospermia (3.3%) or zero post-thaw motility (5.7%). Among the remaining 833 (89.6%) whose sperm was cryostored, 141 (15.1%) subsequently died. Among those surviving their illness (692; 74.4%), material was discarded for 193 men (27.9% of survivors) and material was used for 64 men in 85 treatment cycles aiming to produce a pregnancy using artificial reproductive technologies. This resulted in 29 pregnancies and 39 births; pregnancies were successfully obtained with sperm cryostored for 11 years. Men who were married or had Hodgkin's Disease at the time of cryostorage were more likely to subsequently request sperm usage. Survival analysis indicated that ICSI had superior conception rates (median time 3 cycles) compared with conventional IVF or artificial insemination (median >8 cycles) ( $P < 0.001$ ). Of the 141 men who had died, most had sperm discarded as agreed at the time of storage. Despite written agreement to discard sperm on death, requests to prolong cryostorage after death were received from relatives of 21 men (2.3% of all applicants, 14.9% of deceased). Among the 21 requests to prolong sperm cryostorage post-mortem, most arose in the immediate bereavement period (median 1 week after man's death) and decision-making to accept discarding of sperm was protracted (median 192 weeks). Only in 3 cases had sperm ever been transferred postmortem for use with no known pregnancies. The relatives of men who were married or had leukemia/bone marrow transplantation as reason for cryostorage were significantly more likely to request postmortem maintenance of sperm storage. There were no significant differences in demographic or semen parameters that distinguished either men requesting pre-mortem sperm usage or for whom continued post-mortem storage was sought. Sperm concentration was significantly lower in hemi-orchidectomized men with testicular tumors than among those with other cancers, non-malignant disease or healthy sperm donor controls. Hence elective sperm cryopreservation effectively allows men to preserve their progenitive potential; such fertility insurance should be offered to all men who have not completed family formation and whose fertility is predictably threatened by medical treatments. However usage is sparse, occurring usually within the first 3 years of cryostorage, and rarely after 15 years.

## CALCIUM HOMOESTASIS AND THE CONTRACTILE PROPERTIES OF RAT CARDIAC MYOCYTES ARE DIFFERENTIALLY REGULATED BY OESTROGEN AND TESTOSTERONE

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We have recently demonstrated that there is a marked sex difference in the calcium handling and contractile properties of single rat cardiac myocytes. Cells from female rats have lower baseline, and stimulated, concentrations of intracellular calcium ions ( $[Ca^{2+}]_i$ ), that are associated with a decreased extent of stimulated cellular shortening. These observations may have some implications for the sex-dependent differences in cardiovascular disease and myocardial functioning. We have therefore investigated the roles of the ovaries and testes, and their cognate steroids, oestradiol (E2) and testosterone (T), in the control of these sex-dependent differences.

Adult male and female rats (250-350g) were used in these experiments. Female animals divided into three groups: ovariectomy plus E2 17 $\beta$  (150  $\mu$ g/ml in sesame oil in pellets sc; 1cm/100g rat); ovariectomy plus oil pellets and sham ovariectomy plus oil pellets. Male animals were also divided into three groups: orchidectomy plus crystalline T (1cm silastic capsule sc/100g rat); orchidectomy plus empty pellet, and sham orchidectomy plus empty pellet. Two weeks later, the animals were killed and single cardiac myocytes isolated using enzymatic digestion techniques. The myocytes were loaded with fura-2, and changes in  $[Ca^{2+}]_i$  measured by microspectrofluorimetry under basal conditions and in response to electrical stimulation. The contractile responses of separate individual myocytes to electrical stimulation were measured using a videodetection technique. Between 8 and 15 cells from 5 to 8 rats were used to obtain data for each of the experimental conditions.

When compared with intact control animals, ovariectomy resulted in a 3-fold increase in basal  $[Ca^{2+}]_i$  that was not affected by E2 replacement. The amplitude of stimulated changes in  $[Ca^{2+}]_i$  were increased 2-3 fold following ovariectomy, and E2 replacement restored the amplitude of  $[Ca^{2+}]_i$  to that seen in intact animals. Similar changes in contractile responses were seen, with ovariectomy, increasing the size of the response, and this change being restored by E2. In male animals, orchidectomy result in a 33% decrease in basal  $[Ca^{2+}]_i$ , when compared with intact animals, with the changes being only partially restored by T treatment. Orchidectomy resulted in an approximate halving of stimulated changes in  $[Ca^{2+}]_i$ , and T replacement completed restored values to those seen in intact animals. Likewise, orchidectomy decreased the contractile response of single cardiac myocytes, which was restored by T.

These results indicate that the ovaries and testes, and E2 and testosterone, differentially regulate calcium homeostasis and the contractile responses of cardiac myocytes. As raised  $[Ca^{2+}]_i$  has been associated with pathological responses in a range of cell types, it is possible that some of the increase in cardiovascular morbidity and mortality seen in response to changing sex steroid environments (eg men vs women, pre- vs post-menopause) is associated with sex steroid-dependent changes in cardiac myocyte physiology.

## REVERSAL OF THE OBESE PHENOTYPE OF THE AROMATASE KNOCKOUT (ARKO) MOUSE VIA ADMINISTRATION OF 17 $\beta$ -ESTRADIOL.

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Aromatase catalyses the conversion of androgens to estrogens. The aromatase knockout (ArKO) mouse lacks the enzymatic machinery to perform this conversion and we have previously established that this lack of endogenous estrogen induces an obese phenotype. We explored the hypothesis that administration of exogenous estrogen would rescue the obese state of these ArKO mice. Estradiol was administered via subcutaneous implants containing 0.05mg 17 $\beta$ -estradiol, providing estradiol plasma levels of 50-75pg/ml. Following estradiol administration to 10-week-old female wild type (WT) and ArKO mice for 21 days, gonadal adipose tissue was collected. As expected, gonadal adipose tissue mass, expressed, as a percentage of body weight was significantly greater in ArKO mice compared with WT mice (p<0.05). Following estradiol administration, ArKO gonadal adipose tissue mass was significantly reduced (p<0.001) comparable to WT mice. Gonadal adipocyte volume was determined using Bouins fixed tissue, sectioned and stained with Haematoxylin and Eosin. Results indicate larger adipocytes in gonadal adipose tissue of ArKO mice compared with WT counterparts (p<0.03), followed by a reduction in adipocyte volume (p<0.02) in estradiol treated ArKO gonadal adipose tissue. A similar pattern was observed upon examination of adipocyte number, namely an increase in number of adipocytes isolated by differential centrifugation of collagenase-treated gonadal adipose tissue of ArKO mice compared to WT, and a decrease in numbers following estradiol administration. In order to understand the mechanisms responsible for this effect we examined the transcript levels of factors involved in adipose tissue metabolism. Total RNA was isolated, reverse transcribed and amplified using real-time PCR (Roche LightCycler™). Results indicate an elevation in mRNA expression levels of LPL (p<0.01), leptin (p<0.05) and PPAR $\gamma$  in ArKO mice compared with WT mice, while ER $\alpha$ , PGC1 and UCP1 remained unchanged. In response to estradiol administration, a reduction in mRNA expression levels of LPL (p<0.03), leptin, ER $\alpha$ , PPAR $\gamma$  (p<0.05) and PGC1 was observed in ArKO mice compared to WT mice. These results indicate that the obese phenotype of the ArKO mouse may result in part, from a stimulation of lipogenesis, which is reversed following estradiol administration.

## PRODUCTION OF TESTOSTERONE IN MICE: DIFFERENCES BETWEEN MALE AND FEMALE SUBCUTANEOUS OR OMENTALLY DERIVED PREADIPOCYTES AND THE EFFECT OF DBCAMP

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The deposition of fat around the organs (omentally) is associated with increased risk for the development of certain diseases eg NIDDM and CVD. In human females preadipocytes can produce testosterone and in addition dbcAMP significantly increased the conversion of androstenedione to testosterone (A to T) in omental preadipocytes in culture<sup>1</sup>. It is not known if murine preadipocytes can convert A to T, or if they do so in a pattern similar to humans in gender or site, therefore this work aims to establish an *in vitro* animal model to allow further investigation *in vivo* where human experimentation is not possible. Therefore the aim of this study was to determine if: male and female mouse preadipocytes can convert A to T *in vitro*, if this differs between subcutaneous and omental derived preadipocytes or, in response to dbcAMP.

Preadipocytes were isolated from subcutaneous and omental white adipose tissue derived from adult female or male BALB/c mice. Cells were cultured in Waymouths medium (with 15% fetal calf serum). All cells were cultured with or without the addition of 1mM dbcAMP and exposed to 3.5 $\mu$ M [4-<sup>14</sup>C] androstenedione for 24 hours. Steroids were extracted from media and separated by TLC. Analyses were performed using Mann-Whitney, Kruskal Wallis and Wilcoxon Signed Ranks tests.

Overall female preadipocytes converted more A to T than male. Male subcutaneous preadipocytes converted less A to T than male omental, however there were no significant differences between female subcutaneous and omental preadipocytes. Unlike humans, dbcAMP had no effect on the conversion of A to T in female subcutaneous or omental, but significantly increased the conversion in both male subcutaneous and omental preadipocytes.

These results illustrate for the first time that male and female mouse preadipocytes can convert A to T. Unlike humans, all male mice preadipocytes converted A to T. In addition, dbcAMP had no effect on A to T in female preadipocytes, which is also unlike humans, but significantly increased the A to T conversion in males. This suggests the expression and regulation of the androgenic enzymes in mice may differ between male and female subcutaneous and omental-derived preadipocytes and suggests that further investigation in human gender and site differences is warranted.



## INPUTS FROM GABA AND NA TO GONADOTROPIN RELEASING HORMONE NEURONS IN THE PREOPTIC AREA OF THE SHEEP BRAIN

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Gonadotropin releasing hormone (GnRH) is the brain hormone that is the primary regulator of reproduction. GnRH neurons are found in a region extending from the diagonal band of Broca (dbB) through the preoptic area (POA) with few cells in mediobasal hypothalamus. Synthesis and secretion of GnRH is controlled by feedback regulation of the gonadal steroids and other factors such as stress and season. GnRH neurons do not express steroid receptors at a high level and it is thought that feedback is effected by other neuronal systems that do have high levels of the relevant receptors. In order to establish which neuronal systems are important in the regulation of GnRH synthesis and/or secretion, neuronal systems that express steroid receptors have been studied. Relevant neuronal systems are those that produce gamma-amino butyric acid (GABA) and noradrenalin (NA). Such cells are important targets for steroids and are important regulators of GnRH secretion<sup>1,2</sup>. To determine the extent of direct neural input to GnRH cells, we counted the number of GnRH neurons contacted by nerve terminals that contain either GABA or NA. We also determined whether there is region specificity in regard to which GnRH cells are contacted. Four ewes were killed by Lethabarb overdose and their heads perfused with Zamboni's solution. POA blocks were frozen and 40 µm sections taken and processed for immunohistochemistry. Sections were immunolabelled for GnRH and either GAD (marker for GABA terminals) or dopamine-β-hydroxylase (DBH, marker for NA terminals) using fluorescent labels. Few GnRH cell were seen in the dbB. Initial mapping was done using fluorescence microscopy at 400x to determine which GnRH cells appeared to have DBH-positive and/or GAD-positive terminals in close contact. On this basis, in the medial POA/OVLT, 55% of cells (104/236) received GABA inputs and 26% (49/186) received noradrenergic inputs. In the lateral POA the number of cells receiving GABA inputs was 28% (35/72) and the number receiving NA inputs was 35% (11/31). The extent to which NA provided input to GnRH cells did not differ between the two areas ( $\chi^2=1.23$ ); neither was there a difference in the input provided by GABA ( $\chi^2=0.98$ ). Neurons that appeared to receive inputs were re-examined by confocal microscopy, using a x60 objective lens and optical dissection revealed that only half of the cells with apparent close contacts were in fact possible synapses. In conclusion, evidence of neuronal inputs to cells in the brain is poorly resolved at the light microscopic level, but good resolution is obtained at using optical dissection. In the POA/OVLT, half (54%) of all GnRH cells receive direct input from GABA-producing cells, whereas a quarter (27%) receive input from noradrenergic cells. Nevertheless, estrogen causes secretion of NA in the POA at the time of the LH surge in sheep<sup>3</sup>, pointing to the importance of this system in regulation of GnRH secretion. From these earlier data and the present observations we can infer that a local neuronal network relays input from the noradrenergic cells to the GnRH neurons. On the other hand, the GABA input is probably derived from systems within the forebrain e.g. Bed nucleus of the Stria Terminalis.

## RESPONSES OF PLASMA CONCENTRATIONS OF LEPTIN, INSULIN-LIKE GROWTH FACTOR-I (IGF-I), IGF-II AND IGF-BINDING PROTEINS-1 AND -3 TO GLUCOSE IN HEALTHY YOUNG ADULTS

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IGFs have been implicated in acute insulin-independent glucose clearance and leptin may participate in long-term regulation of glucose turnover. We investigated relationships between the responses of these hormones to glucose in 160 healthy young adult South Australians with normal glucose tolerance aged between 20 and 21 years. Subjects were requested to consume more than 200 g carbohydrate per day for 3 days. They were asked to fast overnight and refrain from smoking and alcohol consumption overnight. In the morning a vein in each arm was catheterised. Blood was withdrawn from one arm before and after (15 occasions) injection of 0.5 g glucose per kg body weight into the other arm. Plasma glucose, insulin, leptin, IGF-I, IGF-II, IGFBP-1 and IGFBP-3 were measured. The effects of i.v. glucose were assessed by RM ANOVA. Glucose injection rapidly increased plasma glucose ( $p<0.0001$ ) and insulin ( $p<0.0001$ ) as expected which returned to baseline levels within 90 and 120 min respectively. Plasma IGFBP-1 fell rapidly after glucose injection ( $p=0.001$ ) and remained suppressed after 60 min as previously reported. Plasma IGF-I ( $p<0.0001$ ) and IGFBP-3 ( $p<0.0001$ ) but not IGF-II levels were reduced by i.v. glucose and remained suppressed for 120 min. Plasma leptin was increased by glucose ( $p<0.05$ ) and remained elevated after 180 min. Basal levels of leptin were positively correlated with those of IGF-II ( $p<0.02$ ) and IGFBP-1 ( $p<0.05$ ). Basal levels of IGF-I correlated positively with those of IGFBP-3 ( $p<0.0001$ ) and negatively with basal IGFBP-1 ( $p<0.01$ ). Furthermore, basal IGF-I was most strongly correlated with plasma IGFBP-1 measured at its nadir 7 min after i.v. glucose ( $p<0.0005$ ). Basal levels IGF-II were also correlated those of IGFBP-3 ( $p<0.0001$ ). Plasma leptin measured before i.v. glucose was positively correlated with IGFBP-3 measured 120 min later ( $p<0.0001$ ). Similarly, basal levels of IGF-II were positively correlated with plasma leptin levels measured 180 min after i.v. glucose. These results show that basal levels of leptin and IGFs are related and determine to some extent their responses to glucose. This suggests that secretions from liver and adipose tissue may form counter-regulatory elements of glucose homeostasis.

## INSULIN-LIKE GROWTH FACTOR-I PREVENTS APOPTOSIS IN GLUCOSE DEPLETED NEURONAL CELLS

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Hypoglycaemia is the major limiting factor of insulin treatment in diabetic patients who aim to achieve normoglycaemia. Glucose is the major source of energy for neurons and decreased availability is particularly damaging to the developing brain. Children who have severe episodes of hypoglycaemia are more likely to have impaired brain function. It has also been shown that neurons undergo cell death when exposed to glucopenic insults. Previous studies have shown that IGF-I is able to protect neuronal cells from apoptosis due to a wide range of insults. Whether IGF-I will protect neurons from glucopenic stress is not known. Two related human neuroblastoma cell lines were used as *in vitro* model systems to examine IGF-I protection of neuronal cells from glucopenic stress: (i) the SK-N-SH-SY5Y cells, express IGF-IR and IGF-I/II enabling autocrine growth and providing resistance to a number of apoptotic stimuli; (ii) the SK-N-SHEP cells with five fold fewer IGF IR, and no detectable IGFs, requiring the presence of exogenous mitogens for survival. The effects of glucopenic stress were examined by culturing cells in serum free media (SF) containing various concentration of glucose (0-25 mM) in the absence or presence of IGF-I (25ng/ml) over a 72 hour period. Three different endpoints were measured; cell number (NBB), cell mitochondrial activity (MTT) and cell survival / apoptosis (ELISA). In both the SH-SY5Y and SHEP cells, the absence of glucose resulted in a dramatic reduction in cell number. In low glucose (0.25-2.5 mM) cell number was decreased for both SH-SY5Y and SHEP cells. Mitochondrial activity was reduced by culture in serum free conditions (25mM glucose) and further decreased at a glucose concentration lower than 2.5 mM, indicating reduced cell survival under these conditions. Addition of IGF-I (25 ng/ml) was able to ameliorate the outcome of the SH-SY5Y cells in both serum free and low glucose conditions. These cells showed a clear response to IGF treatment, with a 2-5 fold increase in both cell number and mitochondrial activity (1-25mM glucose). IGF-I potentially inhibited apoptosis in the SH-SY5Y with a 20% reduction in the absence of glucose and a reduction of up to 60% when glucose was at 1-25mM. The SHEP cells overall showed minimal or no response to IGF-I treatment (25 ng/ml) suggesting inefficient activation of the IGF-IR signaling pathways (mitogenic and antiapoptotic). A differential response to serum free conditions and / or glucopenic insult was also seen in the SH-SY5Y cells, in relation to activation or regulation of glucose transporters. Only GLUT1 was detected by Northern analysis in our neuroblastoma cell lines. GLUT1 expression in the SH-SY5Y cells was up-regulated by lowered glucose levels (0.25 mM) and further increased by IGF-I addition. Analysis of GLUT1 expression in SHEP cell is in progress. Our findings indicate a potential role for IGF-I in protecting neuronal cells subjected to glucopenic stress-induced apoptosis. Likely mechanisms involve both regulation of intracellular glucose transportation and induction of antiapoptotic genes via the IGF-IR. We thus conclude that *in vitro*, IGF-I efficiently protects neurons from glucose deprivation suggesting that IGF-I might have *in vivo* applications in the prevention of hypoglycaemic brain damage.

## PREFERENTIAL INTESTINAL DELIVERY OF LR<sup>3</sup>IGF-I OVER IGF-I IN THE PRE-WEANING VERSUS ADULT RATS.

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During early postnatal development the gastrointestinal tract is highly responsive to exogenously administered LR<sup>3</sup>IGF-I but refractory to IGF-I, in contrast to the mature intestine. Given that LR<sup>3</sup>IGF-I is an IGF-I analogue that binds poorly to IGFFBPs, the response of the intestine is likely to reflect regulation of IGF-I bioactivity by the IGFFBPs. This regulation could occur at the tissue level or via IGFFBPs in circulation. This study examines the second possibility. Circulating IGFBP profiles are markedly different between suckling and adult rats: IGFBP-2 is the main species prior to weaning at which time IGFBP-3 rapidly increases to become the predominant circulating species in adult life. In this study, delivery of exogenous IGF-I peptides to the intestine was measured in pre-weaning (day 19) and adult rats to determine if a correlation exists with circulating profiles of IGFFBPs. Peptides, at a dose known to elicit marked growth responses in the intestine (3.5µg/kg rhIGF-I or LR<sup>3</sup>IGF-I) were spiked with corresponding <sup>125</sup>I-labelled peptide (10x10<sup>6</sup> cpm) and administered intravenously as a bolus to pre-weaning and adult rats (n=6 per group). Tissue distribution of intact peptide was calculated as the TCA precipitable percentage of total radioactivity measured in each tissue after deduction of radioactivity in the samples due to blood content as estimated by haemoglobin levels. Intestinal tissues contained higher levels of intact LR<sup>3</sup>IGF-I than IGF-I in both age groups. However, the ratio of LR<sup>3</sup>IGF-I to IGF-I content was higher in the pre-weaning intestine compared to the adult intestine (1.7 fold in distal SI and 2.7 fold in colon), indicating a difference in delivery of the peptides between the age groups. FPLC analysis of pooled plasma samples examined interactions of exogenous peptides with endogenous circulating IGFFBPs. The profile of exogenous IGF-I in circulation was similar in both age groups. Five minutes after injection the majority of <sup>125</sup>I-labelled IGF-I in plasma eluted in a higher molecular weight peak than control <sup>125</sup>I-labelled IGF-I peptide, indicating IGF-I was bound to endogenous IGFFBPs. In contrast, the elution profile of LR<sup>3</sup>IGF-I in circulation differed with developmental age. In suckling rats when IGFBP-2 levels predominate and IGFBP-3 is minimal, approx 45% of <sup>125</sup>I-labelled LR<sup>3</sup>IGF-I elutes as an unbound monomeric protein. As IGFBP-3 rises to the high levels seen in adult life, less than 25% of plasma radioactivity was in the unbound form and 41% of <sup>125</sup>I-labelled LR<sup>3</sup>IGF-I eluted in a higher molecular weight peak similar to that seen for IGF-I, suggesting binding of the analogue to serum IGFFBPs in the adult. Thus, intestinal delivery of LR<sup>3</sup>IGF-I relative to IGF-I is higher in the pre-weaning rat compared to the adult. This may relate to changes in the circulating IGFBP profile with age, enabling a higher proportion of LR<sup>3</sup>IGF-I to remain in the free form in circulation of pre-weaning versus adult rodents. On the other hand, the majority of exogenous IGF-I appears to be complexed to circulating IGFFBPs in both age groups.

## MODELLING IGF TRANSPORT OUT OF CIRCULATION USING HUMAN UMBILICAL VEIN ENDOTHELIAL CELL MONOLAYERS.

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The insulin-like growth factors (IGFs) are a family of growth factors that are regulated by their binding to a group of high affinity binding proteins called the IGF binding proteins (IGFBPs). In the serum, the bioavailability of the IGFs is primarily regulated by their formation into a ternary complex, composed of IGFs, IGFBP-3 or IGFBP-5 and the acid labile subunit (ALS). It is thought that the size of the ternary complex (150 kDa) inhibits IGFs in the ternary complex from leaving the blood. To study the transport of IGFs, we examined the movement of [ $^{125}$ I]IGF-I across a human umbilical vein endothelial cell (HUVEC) monolayer. HUVEC cells were grown to confluency in transwells containing 0.2 $\mu$ m pores (media : F12 containing 10% FCS, 100 $\mu$ g/ml heparin, 30 $\mu$ g/ml endothelium cell growth factor supplement). When the cells were confluent the monolayer was washed 3x with serum free media after which [ $^{125}$ I]IGF-I, coincubated with IGFBP-3 (10ng/150 $\mu$ l, 1.6nM), ALS (40ng/150 $\mu$ l, 3.1nM) or both, was added to the monolayer. Samples were taken from the bottom well of the transwell after 2 and 4h and the radioactivity counted. Characterization studies of the system showed that [ $^{125}$ I]Des(1-3) IGF-I (an IGF-I mutant that has reduced IGFBP-3 binding) was transported across the monolayer 33% faster than free [ $^{125}$ I]IGF-I (P=0.0042), indicating that endogenous IGFBPs produced by HUVECs are possibly influencing the IGF transport in this system. However over the 4hr incubation time, no free IGFBPs were detectable by ligand blotting nor were cell associated IGFBPs detectable by affinity labeling the HUVEC cell monolayers using [ $^{125}$ I]IGF-I. While the presence of human ALS alone had little effect on the rate [ $^{125}$ I]IGF-I transported across the monolayer, IGFBP-3 and ALS decreased IGF transport by 62.5% (P<0.0001) indicating that the passage of IGF-I was retarded by its interaction with IGFBP-3 and ALS. To further assess the central role of the ternary complex in inhibiting IGF-I transport across the HUVEC monolayer, an IGFBP-3 variant (IGFBP-3 $^{[228]$ MDGEA) which has 10 fold lower affinity for ALS but retains normal IGF-I binding (Firth *et al.*, 1998), was compared to IGFBP-3 for its effect on IGF-I transport. [ $^{125}$ I]IGF-I associated with this mutant was not differentially affected in its transport across the HUVEC monolayer in the presence or absence of ALS. This confirms that the formation of an IGF-I/IGFBP complex with ALS is the key event in preventing trans-endothelial passage of IGF-I. This study thus provides a model system to study the effect that ternary complex has on the passage of IGFs across an endothelial cell monolayer. Future work will evolve around looking at the effects of IGFBP-3 proteolysis on the movement of IGFs across the HUVEC monolayer system when the IGFs are in the ternary complex as well as examining the role of IGFBP-5 ternary complexes on the transport of IGF-I across an endothelial cell monolayer. Supported by NHMRC

## SUPPRESSION OF LUTEINISING HORMONE-RELEASING HORMONE ACTIVITY AND SUBSEQUENT DOWNSTREAM HORMONE EXPRESSION INHIBITS THE GROWTH OF TESTICULAR GERM CELL TUMOURS IN VIVO

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**Aim:** Testicular germ cell tumours appear after the onset of puberty, suggesting the activation of hormonal pathways involved in fertility may be related to tumour development and progression. We sought to determine what effect(s) hormonal manipulation of these pathways would have on the growth of these tumours in vivo.

**Method:** An orthotopic xenograft model of human non-seminomatous germ cell tumour (NSGCT) established previously in our laboratory was used. The luteinising hormone releasing hormone (LHRH) agonist, leuprolide (Lucrin, Abbott Pharmaceuticals) was administered by subcutaneous depot injection to 18 of 24 mice used. After 21 days, during which time LHRH activity was presumed to have been suppressed, NSGCT cells were implanted in the left testis of all mice. Half of the mice treated with leuprolide also received exogenous testosterone via a sustained release subcutaneous implant. All mice were culled 4 weeks after the implantation of tumour cells.

**Results:** Tumour burden is defined as the weight of the tumour-affected left testis expressed as a percentage of the animal's total body weight. Mean tumour burden in mice treated with both leuprolide and exogenous testosterone was significantly smaller than in untreated mice (1.92%  $\pm$  0.36 v. 3.76%  $\pm$  0.36, P < 0.05). Mice treated with leuprolide alone had a mean tumour burden of 2.78%  $\pm$  0.55 but the difference compared to untreated mice did not reach statistical significance.

**Discussion:** Androgen withdrawal is known to cause apoptosis in normal germ cells and this has been related to oestrogen receptor (ER) expression. If malignant germ cells retain this dependence on ER expression to resist apoptosis, perhaps ablation of intratesticular testosterone/oestrogen production would increase the rate of cell death within these tumours. Alternatively, the suppression of follicle-stimulating hormone (FSH) release by the anterior pituitary may be the major factor in reduced tumour growth. Although we do not yet understand the mechanism underlying these data, the importance of such a relationship lies in the potential to use hormonal manipulation as a novel therapeutic strategy in the treatment of testicular cancer. Although current treatment methods cure almost 90% of patients, there are associated long-term side effects of great concern (such as infertility, impotence, hearing loss and secondary malignancy) and a number of patients for whom no current treatment exists. Hormonal manipulation has been used extensively in the treatment of prostate and breast cancer and is known to have minimal toxicity and reversible side effects. This data indicates that similar strategies may be useful in the treatment of advanced or recurrent testicular cancer with the added benefits of reduced toxicity and risk.

**Conclusion:** Testicular germ cell tumour growth is significantly inhibited when LHRH activity and, presumably, subsequent downstream pathways are suppressed. Further research is needed to determine why this association is observed as hormonal manipulation may offer a new approach to the treatment of testicular cancer.

## EFFECTS OF OESTROGENS AND ANTI-OESTROGENS ON GROWTH OF HUMAN DESMOID TUMOUR CELLS

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Desmoid tumours are rare fibroblastic tumours of mesenchymal origin. They occur typically in two settings: sporadically - mainly in healthy young women - and as part of the familial cancer syndrome, Gardner's Syndrome. About 4 new cases occur per million population per year throughout the world. Typically, these tumours are fast growing, resistant to therapy and have a high local recurrence rate (up to 90%) after surgery. Recent evidence has shown that in some cases, despite the absence of demonstrable oestrogen receptor (ER) content, a prolonged response may be obtained to therapy with the ER partial antagonist tamoxifen. To explain these findings, and possibly to contribute to a refinement of options for clinical management, we have studied the cellular and subcellular mechanisms underlying the effects of oestrogens and anti-oestrogens on these tumours. Specifically, we examined growth responses *in vitro* of cultured desmoid tumour cell lines with and without administration of 17 $\beta$ -oestradiol (E2) and the ER antagonists tamoxifen and ICI 182,780 (ICI). Cells were obtained originally from 2 female and 2 male patients and underwent primary culture in phenol red-free DMEM plus 10% fetal bovine serum (FBS) to reach confluence. Cells at passages 1-4 were studied by culturing for 3-5 days in 5% FBS/DMEM with E2 (1-100 nM), tamoxifen (1-1000 nM) or/and ICI (1000 nM).

Typical desmoid morphological characters, elongated spindle shape and whorled pattern, were observed using phase contrast microscopy. Western blotting showed that expression of the  $\alpha$ -subtype of the ER protein was relatively high in the cells of female origin and either very low or absent in those derived from male patients. By contrast, expression of both the  $\beta$ -subtype of ER and of the androgen receptor was low in female and relatively high in male cells. Cells derived from a female patient with familial adenomatous polyposis and an anterior rectus sheath tumour sensitive to tamoxifen treatment responded vigorously to E2 treatment, with a 14% increase in cell numbers at 1 nM, 23% at 10 nM and 25% at 100 nM. In cells derived from two other patients - 1 female and 1 male - with the sporadic form of the disease, E2 induced modest stimulation of growth at 100 nM (7-9% increase,  $p < 0.05$ ), but not at 1 or 10 nM; and in a fourth cell line - from a male patient - E2 had no effect at all and ER $\alpha$  protein was undetectable. Tamoxifen and ICI alone had no effect on cell growth; however, in those cells stimulated by oestrogen both tamoxifen and ICI 1000 nM completely inhibited E2-induced proliferation. The mitogen-activated protein kinase ERK-1 and the early response genes Sp-1 and Egr-1 were strongly expressed by all cells, although Sp-1 expression was higher in the male than in the female cells.

We conclude that desmoid tumours display varying phenotypes. In those forms expressing ER $\alpha$  oestrogens may stimulate tumour growth and anti-oestrogens may block this effect, while other cases may be relatively resistant to the actions of these hormones. Further investigations arising out of these observations may assist in the development of techniques to guide treatment decisions in this condition.

## CHARACTERISATION OF THE EXPRESSION OF THE EXTENDED HUMAN KALLIKREIN (KLK) GENE FAMILY IN NORMAL AND MALIGNANT ENDOMETRIUM

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Endometrial cancer is a hormone-dependent cancer and is the most common malignancy occurring in the female reproductive tract. Despite this, there is little understanding of the molecular basis of endometrial carcinoma (Eca). There is also a need for new approaches in contemporary therapeutic measures for Eca for which the cure rate has not improved in the last 30 years. The tissue kallikreins (KLKs) are a highly conserved multigene family of serine proteases that act on a diverse range of substrates including growth factors, extracellular matrix (ECM) glycoproteins and proteinases. They are also important regulators of ECM degradation and cell proliferation and are regulated by estrogen and progesterone in many systems<sup>1</sup>. KLK1, in particular, has been implicated in normal and malignant uterine function<sup>2</sup>. The human KLK family was recently discovered to have at least 15 members<sup>3</sup>, several of which are expressed in prostate, breast and ovarian cancer<sup>1</sup>. Thus, the aim of this study was to determine which of these genes were expressed in endometrial cancer tissues and cell lines.

Samples of endometrial tumours and normal endometrial curettings were collected from patients of the Royal Womens Hospital. From each sample, total RNA was extracted and reverse transcribed to cDNA for polymerase chain reaction (PCR) and subsequent Southern hybridisation. cDNA from three established Eca tumour cell lines - HEC1B, Ishikawa and KLE - were included as representative of well, moderately and poorly differentiated phenotypes, respectively. We have confirmed the expression of KLK1-3 in the normal endometrium<sup>4</sup> and, in this study, compared their expression profile in Eca. All 15 KLK genes were expressed in both tissue types with varying abundance, although highest levels of expression were seen for KLK6, KLK7, KLK8, KLK10 and KLK13. Except for KLK5 and KLK11, little difference was observed in expression levels for the 15 genes between normal and tumour tissue. KLK5 expression was down-regulated in all tumour samples and the Ishikawa cell line compared with normal endometrial samples and the two other Eca cell lines, HEC1B and KLE, that appear to express KLK5 at high levels. In contrast, KLK11 expression was higher in the Eca samples compared with normal tissue. Immunohistochemistry of K5 and K7 (the putative activator of K5) was also performed on sections of normal endometrium. Preliminary results show K7 protein expression in stroma and glands in the normal endometrium. In contrast, K5 protein showed predominantly glandular staining with some evidence of apical secretion. Similar studies are being performed on Eca tissues.

In this study, we have characterised the expression profiles of the new kallikrein gene family in normal and malignant endometrial tissue. Of interest, two genes, KLK5 and KLK11, show differential expression between tumours and normal tissue. Further studies will be aimed at examining their hormonal regulation and their role in endometrial tumorigenesis

**UNDER-EXPRESSION OF THE B-CHEMOKINE EXODUS-2 GENE IN PAPILLARY CARCINOMA**

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Despite advances in thyroid cancer genetics, the molecular mechanisms underlying thyroid carcinogenesis remains unknown. There is now a limited list of candidate genes, however, none of these show 100% association with thyroid tumours. In an attempt to identify genes that may be linked to thyroid cancer, suppression subtractive hybridisation was performed using RNA from papillary thyroid carcinoma and RNA from normal thyroid tissue obtained from the same individual.

Immediately after surgical removal, thyroid specimens for the study were dissected by pathologists and snap frozen in liquid nitrogen. The cancer samples were taken from the centre of the nodules, while the normal samples were obtained from an area distant from the nodule. Suppression subtractive hybridisation was performed followed by differential screening using the PCR-Select cDNA Subtraction and Differential Screening Kits (Clontech). Ten paired samples were analysed by RT-PCR to verify the difference in expression of candidate genes and northern blot analysis was used to confirm the expression pattern in a further six paired samples.

One of the clones whose expression level was found to be reduced in papillary cancer relative to the corresponding normal thyroid tissue was the Exodus-2 gene. After 25 cycles of amplification, the expression of Exodus-2 was detectable in the 10/10 normal thyroid tissue RNA samples but absent or minimal in the corresponding papillary carcinoma RNA despite comparable amounts of starting mRNA templates. Using northern blot analysis, the higher expression level of Exodus-2 in the normal thyroid RNA compared to the corresponding cancer RNA was evident in 6/6 paired samples.

Exodus-2 is a member of the  $\beta$ -chemokine family, which normally functions to regulate leucocyte migration to inflammatory sites. Some members of the  $\beta$ -chemokine subfamily have recently been shown to have anti-tumourigenic properties. Although this property has not been investigated in Exodus-2, it is tempting to hypothesise that this gene might have some tumour suppressor activity, and subsequent loss of function of the gene (under-expression) could lead to tumour formation.

# POSTER ABSTRACTS

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## EXPRESSION OF Y81 (A SECRETED FRIZZLED RELATED PROTEIN) DURING MAMMARY GLAND APOPTOSIS *IN VITRO*

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The mammary gland undergoes marked proliferation in pregnancy, then after lactation involutes during the post-weaning period via the mechanism of apoptosis, or programmed cell death. Several signalling molecules are up regulated during mammary gland apoptosis, one of which is Y81, a novel apoptosis associated protein which antagonises the Wnt-frizzled pathway. Although the involuting mammary gland is an established model for the study of apoptosis *in vivo*, there are few *in vitro* models to examine mammary gland apoptosis directly. Current mammary gland culture models include whole organ culture (WOC) and explant culture, both of which use hormonal support to maintain mammary tissue in culture for several days. In the absence of such support, however, mammary tissue would be expected to undergo apoptosis. If so, this could provide a model to assess factors that regulate apoptosis during mammary gland involution. In the present study, therefore, an *in vitro* model of mammary gland apoptosis was established and validated by measurement of DNA fragmentation and TUNEL analysis. This model was used to investigate Y81 expression during mammary gland apoptosis using *in situ* hybridisation. Mammary tissue from lactating Wistar rats (n=3) was excised and cultured in serum free media at 37 C under 95% O<sub>2</sub>-5% CO<sub>2</sub> for 0, 2, 4, 6, and 8 hours initially (Experiment 1) and in a second culture (n=3) for 0, 1.5, 3, 4.5, and 6 hours (Experiment 2). Apoptosis, as measured by 3'-end labelling, was significantly elevated from 6 hours (p<0.01) and from 4.5 hours (p<0.01) in the two experiments respectively, when compared to 0 hours (snap frozen). Dying cells were confirmed to be mammary epithelial cells by TUNEL analysis and were particularly prevalent from 6 hours (Experiment 1) and 4.5 hours (Experiment 2). Dead cells were present in fewer numbers at time points before these and were rarely observed at 0 hours. Consistent with the time-dependent increase in apoptosis, there was an associated increase in the Y81 mRNA expression over time. *In situ* hybridisation analysis showed that this expression was cytoplasmic as previously observed for tissue undergoing apoptosis *in vivo*. In conclusion, our results provide support for this novel *in vitro* model to study apoptosis in the lactating mammary gland by confirmation of DNA fragmentation and increased expression of an apoptosis associated gene. Currently, potential inhibitors of mammary apoptosis and the molecular mechanism(s) involved in this process are being investigated.

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## SERTOLI-LEYDIG CELL TUMOR OF THE OVARY, A RARE CAUSE OF PRECOCIOUS PUBERTY IN A TWELVE MONTH INFANT.

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We report a twelve month infant who presented with a 4 month history of isosexual precocious puberty secondary to an estrogenizing Sertoli-Leydig cell tumor of the ovary. Total serum immunoreactive inhibin, and subunits A and B were markedly elevated prior to surgical resection and subsequently decreased 7 weeks later into the normal prepubertal range. Twenty weeks following surgical removal, the patient re-presented with central precocious puberty, inhibin B levels were raised on this occasion, a luteinising releasing hormone stimulation test confirmed central precocious puberty. This is the youngest reported occurrence of this rare sex cord stromal neoplasm. The prognosis of this extremely rare tumor presenting at this early juvenile stage is uncertain. This report illustrates the usefulness of serum inhibin as a tumor marker during therapeutic suppression with leuporelin acetate for central precocious puberty. Analysis of genomic and tumor DNA revealed a normal nucleotide sequence for the LH receptor and the  $G\alpha_s$  gene. In order to understand the molecular pathogenesis of this tumor we analysed mRNA levels for the inhibin A and inhibin B subunits, FSH receptor, LH receptor aromatase, steroidogenic factor-1 and the estrogen receptor  $\beta$  genes. Molecular characterisation reveals the presence of genes specific for granulosa and Leydig cells, the relative expression of these genes in addition to its histologic characteristics suggests that this tumor may result from a dysdifferentiation of a primordial follicle.

## CHARACTERISATION OF TWO GRANULOSA TUMOR CELL LINES COV434 AND KGN

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The ovary produces the gonadal steroids estrogen, androgen and progesterone as well as a number of peptides such as the inhibins and the activins. Development, growth and function of the ovary are controlled by endocrine and paracrine signals. Clearly these may influence the development of ovarian cancer. Cancer of the ovary is the most common fatal gynaecological malignancy. 10% of ovarian cancers are stromal of which granulosa cell tumors (GCT) are the most common. GCT have both morphological and functional similarity to the granulosa cells of the ovarian follicle. We have undertaken an extensive study of genes involved in the FSH signalling pathway to compare their expression in GCT and normal ovarian tissue. Ideally several questions regarding how these tumors arise would be best answered *in vitro*, particularly an assessment of whether second messenger pathways are indeed constitutively activated. Thus far, cell lines derived from human ovarian tumors that express the FSH receptor, aromatase and/or inhibin have been difficult to identify. Two granulosa cell tumor lines, COV434 and KGN, have been reported to express these genes (1,2). Thus the aims of this study are to characterise the profile of expression of known FSH regulated genes in these two cell lines as well as to examine the activation status of cell signalling pathways.

Total RNA from the cell lines COV434 and KGN were reverse transcribed and the cDNA used in RT-PCR assays for the FSH receptor, ER $\beta$ , aromatase, inhibin  $\alpha$ , inhibin  $\beta$ A, inhibin  $\beta$ B, the inhibin receptors P120 and betaglycan, cyclin D2, COX-2 and the LH receptor. Products were visualised on ethidium bromide stained 1.8% agarose gels and subjected to Southern blot analysis. Evidence for activation of second messenger pathways was sought by transfection of reporter constructs in which luciferase expression is driven by specific enhancer elements including cyclic AMP response element (CRE), AP1 response element, glucocorticoid response element (GRE), NF $\kappa$ B response element, and an estrogen response element (ERE).

Genes found expressed in GCT tumors that are also expressed in COV434 and KGN are the FSH receptor, inhibin  $\alpha$ , inhibin  $\beta$ A, inhibin  $\beta$ B, ER $\beta$ , and COX2. Cyclin D2 and the LH receptor are currently being examined. The CRE and AP1 signalling pathways are activated, and we are currently examining the basis of the activation with the objective of identifying which components of these pathways may be mutated.

In order for cell lines to be a valid system to study the physiological regulation of human granulosa cells and/or tumor biology of GCT, these need to have a similar gene expression profile to that seen in granulosa cells and/or GCT. Our results demonstrate the cell lines COV434 and KGN have a pattern of gene expression, which is consistent with that observed in human GCT. This supports our hypothesis that these cell lines are a valid system for dissecting the molecular genetics of human GCT.

## ENDOGENOUS OPIOID PEPTIDES AND THE HPA AXIS IN CHRONIC FATIGUE SYNDROME

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Chronic fatigue syndrome (CFS) is a heterogeneous disorder of unknown aetiology characterised by persistent or relapsing fatigue and other associated symptoms. Previous reports have demonstrated reduced activity of the hypothalamic-pituitary-adrenal axis in subjects with CFS, while others have shown a beneficial effect of exercise. We have previously demonstrated an increase in central opioid tone in highly trained athletes and an increase in CRH in response to prolonged aerobic exercise. A reduction in CRH and/or central opioids may account for some of the observed symptoms in CFS. We therefore hypothesised that subjects with chronic fatigue syndrome would show evidence of a relative cortisol deficiency associated with a reduction in central opioid tone. Twelve subjects fulfilling the International Working Party (1992) definition of CFS and 11 healthy control subjects matched for age and sex took part in the study. All subjects underwent detailed hormonal testing on 3 separate occasions separated by at least 48 hours in a single blinded fashion, receiving naloxone 125  $\mu$ g/kg, ovine CRH 1  $\mu$ g/kg or normal saline placebo by iv bolus over 2 minutes. Plasma ACTH and cortisol were measured at -30, 0, 15, 30, 45, 60, 90 and 120 minutes. Basal activity of the HPA axis was assessed by 0900h cortisol and DHEA-sulphate (on study Day 2), drawn at least 30 minutes after insertion of an iv cannula, and 24h urinary free cortisol. We found no evidence of any alteration in basal activity of the HPA axis in the CFS group. The ACTH response to naloxone was not reduced in the CFS group compared to the controls, and no subject had an abnormal oCRH test. In conclusion, we have not confirmed previous reports of reduced activity of the HPA axis in CFS and find no evidence of reduced central opioid levels as a possible cause for the symptomatology.

## PLASMA ACTH AND URINARY FREE CORTICOIDS IN THE AETIOLOGICAL DIAGNOSIS OF CUSHING'S SYNDROME

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Common tests for the aetiological diagnosis of Cushing's Syndrome (CS) are the measurement of basal plasma adrenocorticotrophic hormone (ACTH) and urinary free cortisol/corticoids (UFC) after high dose dexamethasone. It is expected that the plasma ACTH should be undetectable in ACTH-independent CS. The high dose dexamethasone suppression of 24 hour UFC by 50% is suggestive, and by 90% is diagnostic of pituitary CS.

Our aim was to examine the diagnostic use of basal plasma ACTH and to compare UFC during dexamethasone suppression (2mg 6 hourly) by HPLC and 3 different automated immunoassays without sample extraction. We present our data on 4 patients with pituitary dependent CS and 5 patients with CS caused by adrenocortical tumours.

Basal plasma ACTH levels were obtained on 2 separate days and were measured by chemiluminescence immunometric assay (Nichols Institute Diagnostic, reference range 1.6-11.3 pmol/L). In patients with pituitary CS, the mean plasma ACTH was 19.8 pmol/L (range 10.2-28.7 pmol/L). Only 1/5 patients with adrenocortical tumours had undetectable plasma ACTH of <0.3 pmol/L and the mean for the other 4 patients were 0.8 pmol/L (range 0.4-1.1 pmol/L). While there was a clear separation between the 2 groups, it appears that the current sensitive assays for plasma ACTH may give a detectable though suppressed ACTH levels in adrenocortical tumours.

UFC were measured by HPLC and automated immunoassays on ACS 180, DPC Immulite, and Roche Elecsys. The results of high dose dexamethasone on UFC excretion are shown in the table below :

Type of Assay (reference range)	Urinary Free Corticoids (nmol/day)			
	Basal		% Suppression on 2 <sup>nd</sup> day	
	Mean	Range	Mean	Range
HPLC (<150 nmol/d)	1320	490-1840	94	85-99
ACS 180 (<590 nmol/d)	1800	1400-2300	80	67-94
Immulite 2000 (<900 nmol/d)	3620	2260-4230	89	71->99
Roche Elecsys (not available)	6610	4330-7680	87	69-99

Despite the high baseline UFC on the automated immunoassays, the overall percentage suppression post high dose dexamethasone correlated with that of HPLC. If one expects to achieve more than 90% suppression for Cushing's Disease, HPLC has the highest sensitivity and the ACS 180 the least.

## SPECIFIC CEREBRAL HEMISPHERIC STIMULATION AND ACTIVITY OF THE HPA AXIS

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The human stress response involves the activation of the sympathoadrenal and the HPA axis hormonal responses. Wittling (1990) showed that right cerebral hemisphere activation by visual field stimulation led to a greater salivary cortisol increase than left cerebral hemisphere activation. Vestibular caloric stimulation (VCS) by irrigation of the ear with chilled water causes a generalised activation of the contralateral cerebral cortex. We set out to test the hypothesis that VCS would activate the HPA axis in normal human subjects.

VCS with chilled water commenced at 0 min and continued until horizontal nystagmus occurred. Blood samples for measurement of plasma ACTH and cortisol by RIA were taken at -5, 0, +2, +5, +10, +15, +30, +45, +60 min through a 3 way tap attached to an indwelling cannula. Saliva samples were also obtained (Salivette) for later cortisol analysis by RIA. The experiments were performed in mid-afternoon, a time of low basal activity of the HPA axis, in a quiet, air-conditioned environment in the Clinical Research Rooms of the University Unit at GPH.

In this preliminary report, we present plasma ACTH and cortisol levels measured after VCS of the right ear of a normal male volunteer taking no medication except ranitidine and temazepam. Nystagmus occurred at +1.5 min and VCS was ceased at this time. From a basal level of 3.1 pmol/L, plasma ACTH rose rapidly between 0 and 2 min to a peak of 4.9 pmol/L at 5 min. Plasma cortisol rose promptly from 220 nmol/L to peak at 460 nmol/L at 10 min. ACTH and cortisol returned to basal levels by 30 and 45 min respectively. No stressful events occurred during the experiment.

This experiment demonstrates that VCS is a prompt stimulus to HPA axis activity and its effect rapidly ceases when stimulation is withdrawn. This unique method of activation of the HPA axis has potential to be a very useful technique for studying HPA axis dynamics in humans. Further studies will reveal whether there is cerebral dominance of HPA axis activation. This could be a very useful test to determine if there is a major effect on HPA axis activity in patients with unilateral hemispheric lesion to the hypothalamus or other regions caused by stroke, brainstem tumor or epilepsy.



## PITUITARY-ADRENAL RESPONSES TO ORAL NALTREXONE IN MYOTONIC DYSTROPHY PATIENTS

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Naltrexone (NTX), a competitive opioid antagonist acting at  $\mu$ ,  $\kappa$  and  $\delta$  receptors, exerts a stimulatory effect on the HPA axis which, like naloxone, is probably mediated through hypothalamic CRH release. We previously showed that 50 mg oral NTX caused consistent ACTH and cortisol stimulation in 8/8 normal subjects, but lower doses (25 and 12.5 mg) provided less consistent stimuli. In the majority of myotonic dystrophy (DM) patients, treatment with IV naloxone (and other CRH-mediated stimuli) causes an ACTH hyperresponse, compared to normals. We hypothesised that oral NTX would cause a similar ACTH hyperresponse in DM patients.

In a single-blinded and randomized manner, three adult male DM patients received placebo, 25 mg and 50 mg oral NTX. Blood samples for RIA measurement of plasma ACTH and cortisol were taken through a 3 way tap attached to an indwelling cannula at -15, 0 (time of administration), +30, +60, +75, +90, +105, +120, +135, +150, +165, +180, +195, +210 and +240 min. The experiments were performed in mid-afternoon, a time of low basal activity of the HPA axis, in a quiet, air-conditioned environment in the Clinical Research Rooms of the University Unit at GPH. ACTH responses (pmol/L) in 3 patients and cortisol responses (nmol/L) in 1 patient are shown in the table.

	ACTH $\Delta$ (25 mg)	ACTH $\Delta$ (50 mg)	ACTH $\Delta$ (Naloxone Test)	Cortisol $\Delta$ (25 mg)	Cortisol $\Delta$ (50 mg)
Patient 1	7.9	17.3	12.0	222	393
Patient 2	3.8	6.9	14.0	---	---
Patient 3	3.5	3.3 (or 0)	6.7	---	---
Normal Range	0 - 6.5 (n=7)	1.3 - 7.8 (n=8)	0.8 - 13.2 (n=49)		

Patient 1 had a greater ACTH response than any of the normals to either NTX dose. This agrees approximately with results from a naloxone test (125  $\mu$ g/kg) in this patient, where the ACTH response was at the upper end of the normal range. In addition, the magnitude of the ACTH and cortisol responses appeared dose-dependent. Patient 2's ACTH responses to NTX also appeared dose-dependent, but they were within the ranges observed for normals. This contrasts with Patient 2's response to naloxone, which was above the normal range, categorizing him as a "hyperresponder" to naloxone. Patient 3, a normal responder to naloxone, had a normal response to 25 mg NTX. However, we were unable to determine his response to 50 mg NTX. The ACTH  $\Delta$  of 3.3 pmol/L only occurred at the final sampling point and may not have been a direct consequence of NTX stimulation. ACTH responses to 25 mg NTX peaked earlier than those to 50 mg in Patients 1 and 2, probably due to more rapid absorption of the lower dose. In conclusion, we showed preliminary evidence that NTX may stimulate the HPA axis in DM patients in a dose-dependent manner, and that some patients may have a greater response to NTX than normal controls, supporting a role for CRH in mediating the NTX response. Responses to oral NTX in these patients may show greater variability than those to IV naloxone, due to possible impairment of oral drug absorption in DM. Further NTX testing of patients and normal controls is required.

## EXPRESSION OF PUTATIVE ALDOSTERONE-REGULATED GENES

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The molecular mechanisms by which aldosterone regulates epithelial electrolyte transport in the distal colon and the distal nephron remain to be fully elucidated. The response, which is mediated by the mineralocorticoid receptor (MR), involves direct regulation of gene expression. Primary aldosterone-regulated genes should exhibit an acute (<3 hours) change in mRNA levels in the target tissues. Five genes, sgk, CHIF and the three epithelial sodium channel (ENaC) subunit genes, have recently been shown to be regulated acutely by aldosterone in the mammalian distal colon and/or nephron. In the present study we have extended our analysis to three other putative aldosterone-induced genes. As ENaC plays a well established crucial role in the response to aldosterone, we have examined the expression of two genes encoding putative regulators of its activity. Channel-activating protease (CAP1) is an epithelial serine protease that enhances the activity of ENaC in injected amphibian oocytes. Nedd4-2 is proposed to act in the degradation and thus turnover of ENaC. The third candidate aldosterone target gene is K-ras2, which encodes a G protein involved in multiple signaling pathways. There are two transcripts, A and B, which are generated by alternate splicing; the B isoform is the predominant one in most tissues. The A isoform was found recently to be rapidly upregulated by aldosterone in amphibian cultured cells. The acute response to a single parenteral dose of either aldosterone or dexamethasone was examined *in vivo*. Gene expression was measured by Northern blot analysis in the kidney and distal colon of adrenalectomised rats 0.5 to 4 hours after steroid treatment. The mouse CAP1 cDNA probe was generously provided by Professor Rossier; the Nedd4-2 and K-ras2 probes were amplified by RT-PCR, from rat kidney and colonic RNA respectively, using primers derived from the published sequences. Expression of a ~2.4kb CAP1 transcript (cf ~1.8kb in the mouse) was found in many tissues including the distal colon and kidney. Additional transcripts were observed in several tissues. No response was observed to either chronic or acute elevations of aldosterone or dexamethasone in either distal colon or kidney. The predicted transcript of ~4kb was detected by the Nedd4-2 probe in both kidney and colon, and appeared to show no response to corticosteroid treatment. An additional ~2.5kb transcript was observed in aldosterone-treated colon; this is currently being investigated. The K-ras2 and -2A-specific probes detected a transcript of ~2kb; preliminary analysis indicates upregulation by both steroids in the colon but not the kidney. Although CAP1 represents a novel intraluminal mechanism for the regulation of epithelial sodium transport, this effect would appear to be parallel to, rather than within, the aldosterone-regulated pathway. The apparent upregulation of Nedd4-2 expression by corticosteroids is unexpected, given the putative involvement of this protein in ENaC degradation. Several studies in amphibian systems have provided strong evidence of both regulation of K-ras2A by aldosterone and a role for the K-Ras(A) protein in mediating the aldosterone-induced increase in sodium transport. In view of the broad range of cellular effects of Ras, it is important to determine whether these observations are relevant to mammalian systems.

## Y81 IS UPREGULATED WITH APOPTOTIC CELL DEATH DURING OVULATION

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The process of ovulation is a complex, multi-factorial event which involves the degeneration of a specific area of both the ovarian and follicular surface via the process of apoptosis, a particular form of physiological cell death. Many apoptosis related genes have been identified in the ovary, one of which is Y81, a recently discovered gene that is known to be up-regulated during apoptosis in hormone dependent tissues. The objective of this study was to investigate whether Y81 is also up-regulated with apoptosis during ovulation. To test this hypothesis, ovulation was induced in sexually immature Wistar rats (40-50g) by treatment with subcutaneous injections of 25 IU Pregnant Mare Serum Gonadotrophin (PMSG) and 56 hours later with 8IU Chorionic Gonadotrophin (CG). Ovariectomy was performed at 0, 3, 6, 9, 12, 18 and 24 hours after CG administration. Plasma samples were collected at these time points for progesterone concentration analysis in order to confirm ovulation. Apoptosis was assessed in ovaries using 3'-end labelling of DNA and localised by TUNEL assay. Y81 expression was detected using *in situ* hybridisation. Twenty four hours following CG administration the plasma progesterone levels were significantly higher ( $p < 0.001$ ) than saline treated controls, confirming that hormonal treatment induced ovulation and the subsequent formation of a corpus luteum. Twelve hours after CG injection, there was a 2.5-fold increase ( $p < 0.01$ ) in DNA fragmentation compared to 0 hours. Consistent with the increase in apoptosis, *in situ* hybridisation revealed an increase in Y81 mRNA expression in the thecal cells of pre-ovulatory treated animals. This study has demonstrated for the first time that there is an association between apoptosis and increased expression of Y81 during ovulation in the rat, providing further support for the role of Y81 during apoptosis in hormone dependent tissues.

## ANDROGENS ENHANCE ENDOTHELIAL VASCULAR CELL ADHESION MOLECULE-1 (VCAM-1) EXPRESSION VIA AN ANDROGEN RECEPTOR INDEPENDENT PATHWAY.

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The marked gender difference in incidence and severity of cardiovascular disease is usually attributed to estrogen's protective effects. We recently demonstrated that a key early event in atherosclerosis, the binding of monocytes to the endothelium, is enhanced by a non-aromatisable androgen, dihydrotestosterone (DHT), via increased cellular expression of an important cellular adhesion molecule, VCAM-1. We now demonstrate that DHT mediates its effects on VCAM-1 expression at the promoter level via NF- $\kappa$ B activation, rather than in a classical androgen receptor (AR) mediated pathway.

Luciferase reporter assays demonstrated specific DHT stimulation of VCAM-1 promoter activity by  $130 \pm 9\%$  ( $p < 0.05$ ). As the VCAM-1 promoter has no classical androgen response element but contains regulatory motifs for AP-1, GATA and NF- $\kappa$ B, we assessed DHT effects on transcription factor DNA binding. Electrophoretic mobility shift assays (EMSA) and luciferase reporter assays showed that DHT activated NF- $\kappa$ B and GATA but not AP-1. A VCAM-1 promoter construct lacking the AP-1 motif was induced by DHT by  $138 \pm 12\%$  ( $SD = \pm 0.42$ ,  $p < 0.05$ ) confirming that AP-1 is not required for DHT enhancement of VCAM-1 expression. Blocking AR with a 10-fold molar excess of androgen receptor blocker (hydroxyflutamide, 4 $\mu$ M) did not inhibit DHT-mediated enhancement of VCAM-1 gene transcription, promoter activity or NF- $\kappa$ B activation. Furthermore, co-transfection of AR negative (HeLa) cells with an AR expression vector and a NF- $\kappa$ B luciferase reporter vector showed that DHT decreased NF- $\kappa$ B activation.

## RELAXIN GENE EXPRESSION AND BINDING SITES IN EXTRA-OVARIAN TISSUES OF THE MOUSE

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It is without a doubt, that relaxin is a versatile hormone. First discovered for its role in the relaxation of the pubic ligament during parturition<sup>1</sup>, its 75 year journey of research has led us to identify and postulate roles in the pubic symphysis, uterus, mammary glands, skin, brain and heart. Produced in the ovary, and in some animals, in the placenta, relaxin is believed to act on tissues via specific receptors located on target cells. Mice with a non-functional relaxin gene have been observed to be fertile, have normal pregnancies and produce normal litters<sup>2</sup>. However, abnormal development of the nipple resulted in the mother's inability to suckle their young<sup>3</sup>. Interestingly, relaxin null male mice demonstrated delayed growth and had underdeveloped reproductive tracts<sup>4</sup>. Older male relaxin null mice have higher heart weight<sup>5</sup>. It is increasingly apparent that the roles of relaxin are not just within the reproductive system but also in the central nervous system of the mouse. The aim of this novel study, in the mouse, is to systematically identify extra-ovarian sites of relaxin gene expression and binding. RT-PCR, with specially designed primers that span the intron of the relaxin gene, was used to identify the sites of relaxin gene expression in tissues of the female mouse at various stages of pregnancy. The results demonstrate extra-ovarian relaxin gene expression in tissues of the c57B6J mouse. Although the relaxin receptor has yet to be isolated, receptors are conventionally visualised using radioactively labelled synthetic human relaxin 2 (h2Rlx). In this study, we examined the distribution of relaxin binding sites in a number of extra-ovarian murine tissues, including the uterus, fetus, placenta, cervix, vagina, brain and kidney. These autoradiographic studies have revealed a widespread distribution of relaxin binding sites in specific nuclei of the mouse brain. Furthermore, the results are similar to studies in rat brain where relaxin mRNA and binding sites in specific areas have been found<sup>6</sup>. In summary, the widespread distribution of both relaxin gene expression and relaxin binding sites in the female mouse supports the hypothesis that relaxin has a broad range of functions outside the female reproductive tract.

## A NOVEL TRANSCRIPTION FACTOR BINDS TO THE CRE IN THE CRH PROMOTER.

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Corticotropin releasing hormone (CRH) is a neuropeptide involved in the HPA axis and stress response. It is also expressed in peripheral sites including the placenta. There is a progressive increase in placental CRH production throughout pregnancy with a peak corresponding to labour. Studies suggest that there is a relationship between CRH concentrations and the length of gestation. This is particularly significant in predicting pre term birth. Therefore, elucidation of the molecular mechanisms controlling CRH gene expression is important. Using methods such as site-directed mutagenesis and transfection of primary cultures of placental syncytiotrophoblast cells, key regulatory DNA sequences controlling placental CRH gene expression have been identified (see abstract by BR King et al). The nuclear proteins that interact with these key regulatory sequences have been identified using electrophoresis mobility shift assay (EMSA) and yeast one-hybrid technologies.

These data show that the CRH promoter contains a cAMP regulatory element (CRE) which forms DNA-protein complexes with placental nuclear proteins *in vitro*. To identify placental nuclear proteins which bind the CRE we used a yeast one-hybrid system with the CRE as the target. Screening of a placental cDNA library yielded a cDNA clone, the sequence of which is not homologous with any known transcription factor, but the predicted protein sequence includes a leucine-zipper motif compatible with known transcription factors with DNA binding properties. The capacity for this putative new transcription factor to specifically bind the CRE was confirmed by EMSA. This protein has been named CREAP: cAMP regulatory element associated protein.

To identify the murine homologue of this new transcription factor, the human CREAP cDNA sequence was used to conduct a FASTA search of the NR-EST Mouse database; a non-redundant database containing only murine expressed sequence tagged sequences that is a subdivision of GenBank. A murine sequence was found with 90.8% identity over 750bp. Primers were designed from this sequence and used to conduct RT-PCR of a mouse total RNA panel containing pooled RNA from mouse whole brain, heart, liver, lung, spleen and testis. A 247bp cDNA was amplified from all tissues and was also amplified from a mouse pituitary cell line. From this, a whole brain mouse cDNA library was screened to allow isolation and characterisation of the mouse homologue of the human CREAP transcription factor. This murine CREAP clone will allow genetic experiments to be performed in mice, which will lead to identification of the physiologically important roles for CREAP.

## ESTROGEN REPLACEMENT TO ARKO MICE RESCUES THE OVARIAN PHENOTYPE

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Aromatase knockout (ArKO) mice are infertile and display an age-dependent ovarian phenotype characterised by a block in folliculogenesis, cystic ovaries and the transformation of somatic follicular and interstitial cells to a male phenotype. Studies in the early 1900s reported structures in elderly mice comparable to those observed in our mouse model of estrogen deficiency. The aim of this study was to examine the effects of estrogen replacement on the ovarian phenotype of the ArKO mice. Intact wildtype (WT) and ArKO female mice (n=10-14 mice/group) at 7 weeks of age were given either 0.05mg 17 $\beta$ -estradiol (E), or placebo (P) pellets for 21 days. The effectiveness of the estrogen replacement was confirmed by positive estrogenic vaginal smears (taken daily), reduced serum gonadotropins and increased uterine weight at sacrifice. 3 $\mu$ m Paraffin and 1  $\mu$ m epoxy sections were stained with Mason's Trichrome or Toluidine Blue respectively to examine the morphology. As previously reported, the ovaries of ArKO placebo - treated mice contained distinctively, many oversized (more than 7 layers of granulosa cells) secondary/preantral follicles, some antral follicles, although no corpora lutea. In many cases the granulosa cells within these follicles did not show pyknotic nuclei suggesting that the follicles were not atretic. Conversely there were numerous antral sized follicles, clearly atretic as evidenced by uneven granulosa cell layers coincident with 5-10 pyknotic nuclei within their granulosa cell constituents. Additionally, there were also numerous islands of degenerating follicles containing abnormal somatic Sertoli-like cells. Surrounding these and within the interstitial regions in general were Leydig-like cells. The Sertoli-like and Leydig-like cells were not observed in ovaries of E - treated ArKO mice. These ArKO mice contained many large secondary follicles, the majority of which appeared morphologically healthy (absence of pyknotic nuclei). E - treated ArKO ovaries also displayed an abundance of primordial follicles, as well as polyovular primordial and primary follicles. The estrogen replacement regime reversed the appearance of cystic follicles, but was not sufficient to restore ovulation. In summary, E replacement in ArKO female mice partly restored the morphology of the ovary to that of WT, with the exception of the final stages of folliculogenesis and ovulation. It also reversed the sex-reversal phenotype of somatic cells observed in ArKO ovaries. We conclude that estrogen is essential to maintain the female phenotype and normal folliculogenic processes in the ovaries of mice. Due to the hormonal profile and observed ovarian morphology, ArKO mice may provide a useful model for premature menopause.

## OESTROGEN DEFICIENCY IN THE AROMATASE KNOCKOUT (ARKO) MOUSE LEADS TO LIPID ACCUMULATION IN THE LIVER

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The aromatase knockout (ArKO) mouse lacks a functional *cyp19* gene, the gene which encodes the enzyme aromatase. Aromatase is the enzyme responsible for the conversion of C19 steroids to C18 oestrogens, therefore these mice are unable to synthesize endogenous oestrogens. Initial observations revealed an obese phenotype in the ArKO mice compared to wild type (WT) littermates (Jones *et al* 2000. PNAS 97, 12735-12740). In addition to the obese phenotype it has been observed in older ArKO mice that the livers are pale in colour, with histological examination revealing the accumulation of lipid droplets. To further analyse the liver phenotype we investigated whether or not cholesterol metabolism was perturbed in the ArKO mice. ArKO and WT animals were fed a high cholesterol diet to challenge this pathway. Animals were fed either a normal diet (soy free), 0.2 % or 2 % cholesterol diet for 90 days. Body mass and liver mass were determined. There was a significant decrease in body weight of the ArKO males when fed 0.2 % (p<0.05) and 2% cholesterol (p<0.01) diets compared to the ArKO males on the soy free diet. Similar trends were observed for the ArKO females although it was not statistically significant. The liver weight for the ArKO males showed a trend for decreasing mass with increasing cholesterol diet, whereas that of the WT did not change. The ArKO females however, showed no change in liver weight nor did their WT littermates. Histological examination was used to study the liver morphology. Paraffin sections of the ArKO male livers showed substantial lipid accumulation in animals on the soy free diet, but less so in animals fed the 0.2 % cholesterol diet, whereas the livers appeared normal when the ArKO males were fed the 2% cholesterol diet. There was no lipid accumulation in the WT males. There was no difference seen for the ArKO females in any of the groups. Serum samples were collected and cholesterol, HDL and triglycerides were analysed. There was no statistical difference between any of the groups in serum cholesterol levels, although a tendency for elevated cholesterol in both sexes of the ArKO mice was noted. The HDL levels also showed a trend to elevated levels in the ArKO mice. The triglycerides levels in the ArKO females on the soy free diet were also elevated (p<0.05) compared to ArKO females on the 2% cholesterol diet. A similar trend was seen in the males although it was not statistically significant. To ensure that the animals were eating the diets, a food consumption study was carried out to measure food intake. There was no statistical difference between food intake in any of the groups. Interestingly, these results indicate a sexual dimorphic phenotype with male ArKO mice exhibiting a more severe hepatic steatosis compared to females. This novel finding showed that with increased cholesterol feeding the development of the male hepatic phenotype was severely attenuated. To further understand these exciting results, the expression of transcripts related to liver cholesterol and triglyceride metabolism are currently being investigated.

## THERAPEUTIC INHIBITION OF AROMATASE AND DECREASED ESTROGEN LEVELS FACILITATES CONTINUED GROWTH IN ADULTHOOD AND FINAL HEIGHT

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A 21 year-old adult male presented with pubertal delay and short stature. A macroprolactinoma was diagnosed on MRI/biochemistry. On 10mg bromocryptine daily, his prolactin levels normalised, as did the rest of his pituitary function, except for persisting GH deficiency. Testosterone was within normal range, paralleled by spontaneous initiation of puberty. Initial height was 152.5 cm, with 2cm growth over 10 months since normalization of his gonadal status, with an estimated final height well below the 3rd centile. The paediatric opinion was that the likely gain in final height from the administration of GH would be minimal in view of his advanced bone age (15 years), particularly in conjunction with his chronological age.

Human males with aromatase or estrogen receptor  $\alpha$  gene mutations have delayed epiphyseal fusion and continue to grow in adulthood, despite normal pubertal development and masculinization. This has been attributed to reduced estrogen action. Testosterone may act to promote epiphyseal fusion by aromatization to estrogen, rather than by a direct effect. We wondered whether inhibition of aromatization in this man might delay epiphyseal fusion in order to improve final height, while allowing normal pubertal development. Given that estrogen action in men is poorly understood, we were concerned about the difficulty of predicting side-effects from this therapy. Based on three case studies of de novo mutations, and of the transgenic knockout mouse homologues, possible side-effects might be worsening osteoporosis, hyperlipidaemia and azoospermia. We confirmed baseline osteopaenia [T score -4.03(L2-4), -1.63 (NOF)], but normal lipid profiles and only mild abnormalities in seminal analysis (52 x 10<sup>6</sup>/ml, 50% moderate progression).

**RESULTS** After a full discussion with the patient, GH 3.85mU/day was administered in conjunction with the third generation aromatase inhibitor arimidex, aiming to achieve partial reductions in estrogen levels. After 15 months his height increased by 7.8 cm. He is now currently between the 3<sup>rd</sup> and 10<sup>th</sup> centile in height. Progression of bone age is successfully delayed (15.0 to  $\approx$ 15.9 over 15 months). He has achieved stage 5 puberty with testosterone 18nmol/l. Prolactin remains suppressed. There are no side-effects to date. BMD is stable after initial fall consistent with effects of GH (0'L2-4=0.648g/cm<sup>2</sup>; NOF 0.861g/cm<sup>2</sup>; 6 months: L2-4 0.668g/cm<sup>2</sup>, NOF 0.803g/cm<sup>2</sup>; 12 months: L2-4 0.722g/cm<sup>2</sup>; NOF 0.841g/cm<sup>2</sup>). Seminal analysis remains stable (0': 52 x 10<sup>6</sup>/ml, 50% moderate progression; 12 months: 78 x 10<sup>6</sup>/ml, 70% progression). Lipids are stable (cholesterol 3.3nmol/l). He currently plans to stop after a further 1-2cm in growth.

## ULTRADIAN RHYTHMS IN ADULT ENTIRE AND CASTRATE MALE AND FEMALE MERINO SHEEP

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Leptin is a hormone that is secreted primarily from fat tissue and has a role in the regulation of energy balance. It has been shown to have a diurnal rhythm in both humans (1) and mice (2) although there is no apparent circadian rhythm in sheep (3). Pulses or episodes of leptin release have been observed in entire male and female sheep (3,4), although these are variable between individuals in frequency, amplitude and interval. In both sheep studies there was no effect of feeding on pulse parameters.

In the present study we investigated the pulsatility of leptin release in merino sheep over a 7.5-hour period. Ten-minute samples were collected through a jugular catheter from wethers (n=5), rams (n=6), ovariectomised (OVX) ewes (n=5) and entire ewes (n=6) at days 5 and 12 of oestrus. These animals had free access to feed and water.

Pulse parameters were analysed using the TurboPulsar program. The pulse duration and interval between pulses were similar between all groups (P>0.05). The leptin pulse amplitude differed between groups with rams and day5 ewes having the lowest amplitudes (3.8 & 4.2 ng/ml, respectively) with wethers, day12 ewes and OVX having the highest amplitudes (5.2, 6.05, 6.3 ng/ml, respectively). The maximum concentration of leptin release was 12.4 ng in the OVX and lowest in the rams (7.03 ng).

Differences in pulse frequencies in humans vary from 3.6  $\pm$  0.3 pulses/24 hrs (20 min sampling) (5) to 32.0  $\pm$  1.5 pulses/24 hrs (7 min sampling) (1), with these differences being attributed to variations in sampling regime (1). In adult ewes a 15min-sampling regime revealed a 24 hr pulse frequency of 4.8  $\pm$  1.5 pulses (4). Using a ten-minute sampling protocol over 7.5 hrs, we have found a pulse frequency of 10.4 pulses with mean length of 34.9 min and mean interval of 45.4 minutes. These data show that there is a difference in amplitude between the follicular and luteal phase in Merino ewes (P<0.05) and suggest that the gonads have little influence in the regulation of amplitude of leptin release.

## CAVERNOUS SINUS SAMPLING IDENTIFIES NEARLY RANDOM PATTERNS OF FSH SECRETION IN OVARECTOMIZED EWES.

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Immunoassay analyses of FSH have reported variously pulsatile, non-pulsatile and compositely pulsatile and non-pulsatile modes of release. We postulated that these disparities in part reflect the reduced signal-to-noise ratio inherent in FSH pulse estimation procedures applied to peripheral blood samples, and/or occasional immunological-biological assay inconsistencies. To address these issues, we directly sampled cavernous sinus blood and jugular venous blood concomitantly from ovariectomized sheep every 5 minutes (n=6) and every 1 minute (n=4). Samples from the former were assayed by radioimmunoassay (RIA) and those from the latter by RIA and bioassay (BIO). Waveform-independent discrete peak detection revealed FSH pulses occurring with a mean ( $\pm$ SEM) interpulse interval of  $42 \pm 4.7$  min (5 min data) and every 11 to 13 min (1-min data by RIA and BIO respectively). FSH pulse amplitudes averaged 145-214% of preceding nadir values (set at 100%), but pulsatile secretion constituted only 28% of total secretion. Analysis of the subordinate pattern regularity of FSH release by the approximate entropy statistic disclosed nearly mean-random (equivalent to 1000 randomly shuffled surrogate) FSH time series at both sampling intensities. Synchrony quantification via linear cross-correlation analysis showed significant sample-by-sample co-release of FSH and LH ( $P < 0.0001$ ), which was corroborated by the nonlinear, pattern-sensitive (conditional probability) cross-approximate entropy statistic ( $P < 0.01$  versus random pattern asynchrony). In contrast, albeit non-random ( $P = 0.042$ ), only 13% of discrete FSH and LH pulses were concordant, pointing to strong dissociation of pulse-release mechanisms. We infer that FSH secretion exhibits diminutively pulsatile and prominently basal/non-pulsatile features, which approximate random secretory output (high-entropy features). Rapid pulsatile production of FSH was evident in the profiles of animals sampled every minute. FSH and LH release showed joint sample-by-sample synchrony by both linear and non-linear statistical measures, indicating pattern coordination despite sparse synchrony of burst-like (pulsatile) output. Intermittent, regular and concordant FSH and LH pulses are most probably due to pulsatile gonadotropin releasing hormone (GnRH) input, but other non-GnRH mechanisms could account for independent FSH pulses.

## COMPLEX CROSSREACTIVITY OF BINDING PROTEINS FOR INHIBIN, ACTIVIN AND TRANSFORMING GROWTH FACTOR (TGF)-B.

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Inhibin opposes the actions of activin in several, but not all, activin-responsive cell types. Both inhibin and activin are structurally related to TGF- $\beta$ . To stimulate cells, activin and TGF- $\beta$  bind to their respective type II receptors (70-75 kDa) and each complex then recruits complementary type I signalling receptors (50-55 kDa). The nature of inhibin signalling is less clear, but inhibin can recruit several different binding proteins, including activin type II receptors (ActRII), into high affinity complexes with TGF- $\beta$  type III receptor, betaglycan, and thus can block activin signalling (1,2). To test the hypothesis that dimers containing a common subunit will compete for subunit-specific binding proteins, we have compared inhibin A (a.bA), activin A (bA.bA) and TGF- $\beta$ 1 (b1.b1) binding and affinity labelling in mouse Sertoli (TM4) cells, which express betaglycan and ActRII (2).

TM4 cell cultures were incubated with recombinant human (rh) [125I]inhibin A, [125I]activin A or [125I]TGF- $\beta$ 1 in HEPES-buffered DMEM:F12 medium containing 0.1% BSA and protease inhibitors in the absence or presence of unlabelled rh inhibin A, activin A or TGF- $\beta$ 1 for 4 h at 24 C. Bound hormone was either recovered directly in 0.1% Triton X-100 (binding studies), or was chemically crosslinked to cell surface proteins using bis-sulphosuccinimidyl suberate (BS3, 0.25 mM) during 30 min at 4 C, then was extracted with 0.50 ml of 1% octyl- $\beta$ -D-glucopyranoside. Affinity labelled proteins in 0.10 ml aliquots of extract were separated by non-reducing 7.5% SDS-PAGE and analysed by autoradiography. The table below summarizes results of binding and affinity labelling studies on TM4 cells.

Radioligand	Competitor IC50 (nM)/% inhibition			Deduced sizes of competed proteins (kDa)		
	INH A	ACT A	TGF- $\beta$ 1	INH A	ACT A	TGF- $\beta$ 1
[ <sup>125</sup> I]INH A	0.2 / 80	$\gg$ 10 / nd	0.9 / 50	45,65,75,115, 125,>200	-	45,65,75,115,>200
[ <sup>125</sup> I]ACT A	$\gg$ 10 / nd	0.15 / 25	$\gg$ 4 / nd	-	<45	-
[ <sup>125</sup> I]TGF- $\beta$ 1	$\gg$ 10 / nd	$\gg$ 10 / nd	<4 / $\gg$ 30	-	-	50,65,75,>200

nd = none determined at the highest tested concentration of competitor

These results show that each unlabelled ligand competed with its homologous radioligand for binding to Sertoli TM4 cells but, in all but one case, was ineffective against heterologous radioligands. In particular, activin A competed poorly with [125I]inhibin A for binding to TM4 cells. The structurally less closely related TGF- $\beta$ 1 (b1.b1) provided the exception, competing with [125I]inhibin A for binding to most species, including the core protein and glycosylated forms of betaglycan (115 and >200 kDa, respectively). However, [125I]TGF- $\beta$ 1 binding to these betaglycan forms and other proteins in TM4 cells was insensitive to inhibin. The 75 kDa protein that bound [125I]inhibin A bound little or no [125I]activin A, suggesting it is not ActRII, but a similar sized protein bound [125I]TGF- $\beta$ 1. It is concluded that binding of these ligands does not simply reflect the subunit composition of each dimer. Supported by the NH&MRC of Australia.

## FOLLISTATIN EXPRESSION IN MATERNAL AND FOETAL LIVER ACROSS GESTATION IN THE SHEEP

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Follistatin is a monomeric protein first isolated from follicular fluid that occurs in at least two different alternatively spliced variants, each with varying degrees of glycosylation. Subsequently, follistatin was shown to be a binding protein of both activin and inhibin, both of which are found in many tissues and organs and are potent regulators of FSH synthesis and secretion. Evidence from studies of liver after partial hepatectomy show that follistatin mRNA is upregulated and exogenous intravenous follistatin promotes more rapid regrowth of the liver than untreated animals (1). The aim of this study was to investigate follistatin concentrations and localisation in the liver during development in the sheep. Follistatin was measured in this study using an assay which is not interfered with by activin as previously described (2).

In this study, follistatin was measured in merino sheep liver samples taken from both maternal and foetal sheep at different stages of gestation. Samples were taken from groups of up to 10 animals at days 50, 75, 125 and 140 of gestation. Maternal expression of follistatin did not significantly change ( $P > 0.05$ ) across gestation with a mean of  $0.46 \pm 0.06 \mu\text{g/g}$  of liver tissue. Foetal expression of follistatin declined across gestation  $12.15 \pm 1.36 \mu\text{g/g}$  of tissue at day 50 to  $0.33 \pm 0.04 \mu\text{g/g}$  just prior to parturition at 140 days. In addition, immunohistochemical studies showed specific staining of Follistatin in the epithelial cells of foetal liver capillaries. No staining could be observed in any of the larger blood vessels in foetal liver or in maternal liver tissues.

In conclusion, we found significant differences in the concentration of follistatin in foetal livers between early and late gestational development. The high levels of follistatin in early gestation may modulate angiogenesis in the rapidly growing liver. This may account for its subsequent decrease over gestation and the minimal levels observed in adult liver tissue.

## CHARACTERISATION OF INHIBIN FORMS AND THEIR MEASUREMENT BY AN INHIBIN $\alpha$ SUBUNIT ELISA IN SERUM FROM POSTMENOPAUSAL WOMEN WITH OVARIAN CANCER

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Previous studies (1) had shown that inhibin assays which detected all inhibin forms containing the  $\alpha\text{C}$  region of the inhibin  $\alpha$  subunit were more sensitive in detecting certain ovarian cancers (granulosa cell tumours and mucinous carcinomas) than inhibin assays which detected either the  $\alpha$  subunit or fragments (Pro- $\alpha\text{C}$ ) or dimeric inhibin A and B forms. The aim of this study was to characterise the molecular wt forms of inhibins A and B and its free  $\alpha$  subunit present in serum from women with ovarian cancer as a basis for developing improved monoclonal antibody-based inhibin assays for monitoring ovarian cancer. Thus three new inhibin  $\alpha$  subunit ( $\alpha\text{C}$ ) ELISAs were developed utilising monoclonal antibodies directed to three non-overlapping peptide regions of the  $\alpha\text{C}$  region of the inhibin  $\alpha$  subunit. In order to characterise serum inhibin molecular wt forms present in women with ovarian cancer, existing inhibin immunoassays (inhibin A, inhibin B, Pro- $\alpha\text{C}$ ) and the new  $\alpha\text{C}$  ELISAs were applied to sera from women with granulosa cell tumours and mucinous carcinomas previously fractionated using a combined immunoaffinity chromatography, preparative SDS-PAGE and electroelution procedure. The distribution and molecular size of dimeric inhibins and  $\alpha$  subunit detected were consistent with known molecular wt forms of inhibins A and B and inhibin  $\alpha$  subunit and their precursor forms present in serum and follicular fluid from healthy women. The  $\alpha\text{C}$  ELISAs recognised all known forms of inhibin and the free inhibin  $\alpha$  subunit, although differences between  $\alpha\text{C}$  ELISAs were observed in their ability to detect high molecular wt forms. To assess which of the  $\alpha\text{C}$  ELISAs was preferred in application to ovarian cancer, the  $\alpha\text{C}$  ELISAs were applied to serum from a range of normal postmenopausal women ( $n = 61$ ) and postmenopausal women ( $n = 152$ ) with ovarian (serous, mucinous, endometrioid, clear cell carcinomas and granulosa cell tumours) and non-ovarian (breast and colon) cancers. Despite differences in their ability to detect high molecular wt forms of inhibin, the  $\alpha\text{C}$  ELISAs showed similar sensitivity and specificity indices in the detection of mucinous carcinomas (84%, 5%) and granulosa cell tumours (100%, 5%) when compared to earlier inhibin RIA or polyclonal antibody-based immunofluorometric assays (1). A combination of the  $\alpha\text{C}$  ELISAs with the CA125 assay, an ovarian tumour marker which has a high sensitivity and specificity for other ovarian cancers (serous, clear cell, endometrioid), resulted in an increase in sensitivity/specificity indices (92-95%, 5%) for all ovarian cancer groups. These new monoclonal antibody-based inhibin  $\alpha\text{C}$  ELISAs now provide practical and sensitive assays suitable for evaluation as diagnostic tests for monitoring ovarian cancers.

## THE EFFECTS OF TESTOSTERONE AND COMBINED TESTOSTERONE + PROGESTIN TREATMENT ON TESTICULAR AND SERUM ANDROGENS IN NORMAL YOUNG MEN<sup>†</sup>

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Testosterone (T) treatment suppresses gonadotropin levels and sperm counts in normal men but the addition of a progestin may improve the efficacy of hormonal contraception. The aim of this study was to investigate the effects and time-course of T ± progestin treatment on intratesticular and serum androgens [T, dihydrotestosterone (DHT) and 5 $\alpha$ -androstane 3 $\alpha$ ,17 $\beta$ -diol (Adiol)] in normal men. A related study has examined serum gonadotrophins, sperm counts and testicular germ cells in the same men. T enanthate (TE, 200mg/im weekly) ± depot medroxyprogesterone (DMPA, 300mg/im once) was given to normal men for 2, 6 or 12 weeks (n=5/group) prior to vasectomy and testicular biopsy. A control group of untreated men (n=5) was also included. Intratesticular T (iT), DHT (iDHT) and Adiol (iAdiol) were extracted, separated by HPLC, quantitated by RIA as previously described<sup>2</sup> and expressed as ng steroid/g wet weight tissue. Serum T was determined by a commercial immunofluorometric assay.

mean (SD)	Control	2wk TE	6wk TE	12wk TE	2wk TE+DMPA	6wk TE+DMPA	12wk TE+DMPA
iT (ng/g)	643 (116)	33.6 (24.9)	13.4 (4.8)	14.7 (2.7)	14.2 (1.8)	13.2 (4.6)	15.9 (2.2)
iDHT (ng/g)	6.4 (2.4)	6.0 (5.4)	3.3 (1.6)	3.7 (1.9)	1.7 (1.2)	2.8 (1.7)	4.5 (2.7)
iAdiol (ng/g)	6.9 (3.2)	4.5 (3.5)	2.8 (1.4)	4.0 (2.5)	3.7 (2.6)	3.7 (3.0)	3.8 (3.8)

In control men iT levels were 100 fold higher than those of iDHT and iAdiol. In response to treatment, serum LH levels fell to less than 1% of baseline. iT levels fell to 5.2% and 2.2% of the control group by 2 weeks of TE or TE+DMPA treatment respectively and were significantly lower in the TE+DMPA men compared to the TE men (p<0.05). Serum LH levels were also significantly lower (p<0.05) in the TE+DMPA group at this time. By 6 and 12 weeks, iT levels were ~2% of control and were similar in both the TE and TE+DMPA-treated groups. Despite the marked decrease in iT levels, there were no significant changes in the levels of the 5 $\alpha$ -reduced androgens iDHT and iAdiol in any of the control or treatment groups by 1-way ANOVA (DHT p=0.21, Adiol p=0.50).

In conclusion, the withdrawal of serum LH results in a 98% reduction in iT levels without a change in the testicular content of 5 $\alpha$ -reduced androgens whose levels are maintained by either their enhanced local 5 $\alpha$ -reductase activity or production in extra-gonadal tissues followed by passive diffusion into testis. Whether these residual androgen levels influence the state of spermatogenesis is the subject of further study.

## TESTOSTERONE AND SPERMATOGENESIS IN A TRANSGENIC MOUSE MODEL EXPRESSING AN ACTIVATING MUTATION OF THE FOLLICLE STIMULATING HORMONE RECEPTOR

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Spermatogenesis requires follicle stimulating hormone (FSH) and lutenising hormone (LH) binding to specific cell surface receptors in the Sertoli and Leydig cells of the testis, respectively. However, in 1996 Gromoll *et al* described a hypophysectomized patient, who, despite the absence of FSH and LH, maintained spermatogenesis and paternity on testosterone (T) replacement alone; his lowest serum T levels however appeared to be higher than castrate levels. A constitutively activated mutation of the FSH receptor (FSHR+) was identified (G→A, Asp→Gly substitution in 3rd IC loop of the receptor, homologous to activating mutations in TSHR and LHR) but direct proofs of its effects have not yet been reproduced experimentally. We created a transgenic mouse expressing this putative human FSHR+, to establish an in vivo model and test the proposed FSHR+ effects on the testis and spermatogenesis in both gonadotropin deficient (hpg) and control backgrounds.

FSHR+/hpg mice showed increased testis size and sperm production, compared with hpg controls. T alone can induce spermatogenesis on the hpg background, and FSHR+/hpg mice had marginally higher serum T (4.41 ± 0.51 nM, n = 20) than controls (3.69 ± 0.53 nM n = 10), resembling the original case. This increase might be due to T production from either the adrenals or testis.

To establish the origin of T in FSHR+ mice, serum T was measured in castrated FSHR- (1.37 ± 0.17 nM, n = 7) and FSHR+ littermates (1.39 ± 0.20 nM, n = 7), suggesting that adrenal contribution to circulating T in FSHR+ mice is negligible.

To determine whether Leydig cells of FSHR+ mice expressed functional FSH receptors, Leydig cells were isolated from non-hpg FSHR+ mice and stimulated with recombinant hFSH or hCG (an LH analogue).

	Testosterone (pg/10 <sup>4</sup> Leydig cells)	FSHR-	FSHR+
Basal (n = 6)		649 ± 19	828 ± 70
+100 ng/ml hFSH (n = 6)		650 ± 55	636 ± 58
+1000 ng/ml hFSH (n = 6)		654 ± 82	554 ± 83
+5000 pg/ml hCG (n = 6)		8066 ± 58	7746 ± 42

We conclude that any excess circulating T in FSHR+/hpg mice is likely to be of testicular origin, but is unlikely to be due to FSHR expression on Leydig cells. Further studies of the spatial distribution of FSHR+ are warranted. We conclude any anomalous T production in FSHR+ mice is most likely due to Sertoli cell mediated paracrine effects.



## STEREOLOGICAL EVALUATION OF TRANSGENIC FSH ACTIONS ON MOUSE SPERMATOGENESIS

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Spermatogenesis is dependent on the pituitary gonadotrophins FSH and LH. Identifying the specific effects of FSH *in vivo* has been difficult due to the intimate relationship between LH and FSH actions and inherent limitations in classical research paradigms. To elucidate the role of FSH in testicular physiology we recently created a transgenic (tg) mouse model by combining tg human FSH expression with the gonadotrophin-deficient background of the hypogonadal (hpg) mouse. This unique model allows the evaluation of specific FSH bioactivity in the absence of LH and testosterone (T). Transgenic FSH/hpg males of founder line±6 expressed consistent serum hFSH levels ( $3.89 \pm 0.48$  IU/L), with a 5-fold increase in testis weights relative to non-tg hpg controls ( $p < 0.001$ ). Histological examination of enlarged FSH/hpg testes revealed the presence of post-meiotic spermatids, whereas spermatogenesis in hpg controls did not progress beyond meiotic pachytene spermatocytes (PS). Stereological analysis revealed that tg FSH stimulated a 2-fold increase in Sertoli cell (SC) numbers ( $p < 0.002$ ) with or without T treatment, and significantly expanded germ cell populations (see table). Spermatogonia (Sg) and spermatocyte numbers were increased by FSH actions alone ( $p < 0.002$  and  $p < 0.003$  respectively), with a 26-fold increase in meiotic PS. The presence of round spermatids (RS) and sparse numbers of elongated spermatids (ES) showed that FSH induces meiotic completion and a small degree of early spermiogenesis. By comparison, T treatment of hpg and FSH/hpg mice resulted in more developed testes with gonadal weights increased 3 and 5-fold respectively relative to FSH/hpg testes. The increased numbers of spermatocytes and haploid spermatids in hFSH/hpg+T testes demonstrates additive or synergistic FSH and androgen effects, particularly on the PS and post-meiotic germ cells. These results also confirm the vital role that androgens play across the whole spermatogenic cycle, in particular during post-meiotic germ cell development. In conclusion, these results show that FSH alone stimulates SC proliferation and plays an important role in promoting early and meiotic germ cell maturation. Combined with androgens, the FSH activity appears to extend to a synergistic effect on subsequent spermatid development. Thus, FSH enhances both the pre- and post-meiotic germ cell capacity of the testes.

	SC $\times 10^6/\text{testis}$	Sg $\times 10^6/\text{testis}$	early 1 <sup>o</sup> Sc $\times 10^6/\text{testis}$	PS $\times 10^6/\text{testis}$	RS $\times 10^6/\text{testis}$	ES $\times 10^6/\text{testis}$
hpg (n=5)	$1.2 \pm 0.1^a$	$1.0 \pm 0.2^a$	$0.6 \pm 0.1^a$	$0.1 \pm 0.03^a$	$0^a$	$0^a$
hFSH/hpg (n=5)	$2.4 \pm 0.2^{bc}$	$2.6 \pm 0.3^b$	$2.7 \pm 0.5^b$	$2.1 \pm 0.5^b$	$1.6 \pm 0.5^b$	$0.2 \pm 0.1^a$
hpg +T (n=5)	$2.1 \pm 0.1^c$	$1.6 \pm 0.1^c$	$3.2 \pm 0.4^{bc}$	$4.3 \pm 0.4^c$	$12.2 \pm 0.3^c$	$10.8 \pm 1.2^b$
hFSH/hpg +T (n=6)	$2.6 \pm 0.2^b$	$2.4 \pm 0.3^b$	$4.6 \pm 0.6^c$	$8.9 \pm 1.0^d$	$19.1 \pm 1.8^d$	$17.3 \pm 2.9^b$

a,b,c,d In each column, means with unlike superscripts are significantly different.

## PREVALENCE OF GROWTH HORMONE DEFICIENCY IN A TERTIARY REFERRAL CENTRE

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We aimed to document known and predicted prevalence of Growth Hormone (GH) deficient (GHD) patients who attended our tertiary referral centre to predict future resource implications. A retrospective survey is in progress on all identifiable pituitary patients who attended our out-patient department between 1985 and 2001. All patients with possible GHD were included; patients with medically-treated microprolactinomas were excluded. Severe GHD was defined as a peak serum GH level  $< 5$  mU/L in response to adequate hypoglycaemia. Demographic details, initial diagnosis, treatment, pituitary hormone status and serum IGF-I were assessed.

A total of 230 adult patients were included, 82% attending the outpatient department since July 2000. Between 1990 and 2001, 29% (66/230) patients had insulin tolerance tests (ITT): 62% (41/66) had severe GHD, 24% had normal responses (peak GH  $> 20$  mU/L), and 14% had an intermediate response. In these patients with ITT results, the prevalence of severe GHD in patients with 4, 3, 2, 1, or no other pituitary hormone deficits (i.e. ACTH, TSH, Gonadotrophins, or ADH) was 100% (6/6), 84% (22/26), 50% (2/4), 40% (3/7) and 0% (0/9), respectively. The prevalence of severe GHD in patients with serum IGF-I concentration below the 5<sup>th</sup> centile for age (Bioclone IGF-I assay; manufacturer's reference range) was 100% (13/13). In contrast 46% (12/26) of severe GHD patients had a normal age-specific serum IGF-I concentration. The prevalence of severe GHD in patients treated with transphenoidal hypophysectomy was 39% (9/23), 2 operations was 60% (2/3), pituitary irradiation alone was 100% (3/3), surgery and irradiation was 94% (15/16), 2 operations and irradiation 60% (4/5), irradiation and any other treatment 92% (22/24) and no treatment 60% (6/10).

Based on, a) the rate of other pituitary hormone deficiency, b) low serum IGF-I concentration and c) treatment modality, we predict that the total number of patients with severe GHD in our total clinic population would be 120 (53%), 104 (45%), and 140 (61%), respectively. Assuming our clinic covers a geographic area with 1.5 million people, we estimate there may be 3,527 similar pituitary patients in Australia, with between 1,587 to 2,151 patients with severe GH deficiency.

We conclude, a) the prevalence of severe GH deficiency in pituitary patients attending a tertiary referral centre is high, b) whilst the ITT is the current diagnostic test of choice, a low age-specific serum IGF-I concentration may be diagnostic, and c) patients receiving radiotherapy are at high risk of developing GH deficiency.

## PHYSIOLOGICAL AND PHARMACOLOGICAL REGULATION OF 20-KILODALTON GH IN HUMANS

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The 20-kDa GH (20K) arises from alternative splicing of the GH primary transcript, with deletion of residues 32-46 of the full-length 22-kDa isoform (22K). It has growth-promoting and lipolytic activities similar to those of 22K, but weaker diabetogenic action. Little is known about the regulation of 20K secretion. Using ELISAs specific for the 2 isoforms (Nihon Schering KK, formerly Mitsui Pharmaceuticals Inc)<sup>1</sup>, we compared serum levels of 20K and 22K in studies of (i) spontaneous secretion over 24h (20-min sampling) in 39 normal subjects, 14 acromegalic and 23 GH-deficient patients; (ii) provocative testing by insulin-induced hypoglycemia in 12 normal subjects; (iii) sex steroid administration to 6 hypogonadal men and 8 postmenopausal women; (iv) octreotide treatment of 8 acromegalic patients; (v) recombinant 22K (0.1 U/kg) administration to 6 normal men.

Mean 24h concentrations of 20K were significantly higher in acromegaly ( $682 \pm 276 \text{ ng/L}$ ; mean  $\pm$  SE;  $P < 0.0005$ ) and lower in GH deficiency ( $10 \pm 4 \text{ ng/L}$ ;  $P < 0.0001$ ) than in normal subjects ( $47 \pm 5 \text{ ng/L}$ ). However, the ratios of 20K to 22K were not different between the 3 groups ( $5.9 \pm 0.8\%$ ,  $7.3 \pm 2.4\%$  and  $6.9 \pm 0.8\%$  respectively). In normal subjects, 20K was secreted in a pulsatile manner throughout the day, with peaks coincided with those of 22K. Deconvolution analysis revealed that 20K was co-secreted with 22K, and the circulating half-life of 20K was significantly longer than that of 22K ( $18.7 \pm 0.8$  vs  $14.7 \pm 0.8 \text{ min}$ ;  $n=8$ ;  $P < 0.02$ ). Insulin-induced hypoglycemia, androgen and oral oestrogen caused a parallel increase in 20K and 22K, with no significant change in the 20K/22K ratio. Octreotide rapidly suppressed both isoforms in acromegalic patients. In normal subjects administered recombinant 22K, 20K fell from  $57 \pm 41 \text{ ng/L}$  at basal to levels below the assay detection limit ( $7.5 \text{ ng/L}$ ) by 4-5h for up to 12h. In summary, 20K circulated at a constant proportion of 22K during spontaneous and pharmacologically manipulated secretion, suggesting co-regulation of the 2 isoforms. Serum 20K was acutely reduced by administration of exogenous 22K, suggesting rapid negative feedback regulation on pituitary release. Measurement of 20K may hold promise as a marker of exogenous 22K administration. (Supported in part by the NHMRC of Australia)

## TESTOSTERONE ENHANCES THE ANABOLIC EFFECT OF GROWTH HORMONE

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Growth hormone (GH) and testosterone (T) are both potent anabolic hormones. It is not known whether the two hormones interact to positively regulate protein metabolism. To address this question, we have investigated the impact of GH alone and of combined GH and T on whole body protein metabolism in hypopituitary men.

Seven subjects were randomised to receive either GH (1.5 IU/d) or GH+T (250mg IMI every 3 weeks) for one month, and then crossed over to the alternate treatment for the second month. Plasma levels of T and IGF-I were measured, and protein turnover studies undertaken before and at the end of each treatment. Protein turnover was studied using a 3 h primed constant infusion of 1-<sup>13</sup>C]leucine, from which rates of leucine appearance (Ra, an index of protein breakdown), leucine oxidation (Lox, an index of irreversible oxidative loss of protein) and non-oxidative leucine disposal (NOLD, an index of protein synthesis) were estimated. Lox and NOLD are expressed as a fraction of Ra. Results were compared to 13 normal subjects.

	IGF-I (nmol/L)	Testosterone (nmol/L)	Ra (mmol/min)	Lox (% of Ra)	NOLD (% of Ra)
Baseline	7.4 $\pm$ 1.0	2.6 $\pm$ 0.6	124.1 $\pm$ 14.1	31.2 $\pm$ 2.1	68.8 $\pm$ 2.1
GH	24.2 $\pm$ 2.7*	1.8 $\pm$ 0.7	138.8 $\pm$ 7.3	26.3 $\pm$ 1.9*	73.7 $\pm$ 1.9*
GH+T	26.4 $\pm$ 2.7*†	12.2 $\pm$ 1.9*†	121.8 $\pm$ 7.7	20.1 $\pm$ 1.2*†	79.9 $\pm$ 1.2*†
Control	16.5 $\pm$ 1.3		122.0 $\pm$ 8.1	21.8 $\pm$ 0.9	78.2 $\pm$ 0.9
* p<0.05 vs. baseline		† p<0.05 vs. GH		p<0.05 vs. controls	

At baseline, Lox was higher and NOLD lower in hypopituitary subjects. Administration of GH significantly increased plasma IGF-I, reduced Lox and increased NOLD. Addition of T to GH resulted in an increase of T into the normal range, a further increase in IGF-I, a further reduction in Lox and further stimulation of NOLD. Neither GH nor GH+T altered leucine Ra.

In summary, GH replacement in hypopituitary adults increased circulating IGF-I, reduced irreversible oxidative protein loss and increased protein synthesis. Concurrent testosterone replacement further amplified these effects. We conclude that testosterone, in physiologic levels, enhances the protein anabolic effect of GH, mediated in part through increased IGF-I production. (Supported by Lilly & NHMRC of Australia).

## AGE-DEPENDENT REGULATION OF THE ACID-LABILE SUBUNIT AND INSULIN-LIKE GROWTH FACTOR-I DURING SHORT-TERM FASTING AND RE-FEEDING

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The acid-labile subunit (ALS) plays an important role in regulating the bioavailability of insulin-like growth factor-I (IGF-I). Both of these proteins are growth hormone (GH)-dependent, age-dependent, and are mainly produced by hepatocytes in the liver. Furthermore, it is well known that circulating levels of ALS and IGF-I decrease during fasting in both humans and animals. The aims of this study were to investigate a) age-related differences in the regulation of ALS and IGF-I gene expression and serum protein levels during short-term fasting and re-feeding, and b) whether age-dependent responses of the GH-signalling pathway might explain differences in response. Juvenile (4 week) and adult (10 week) Sprague Dawley rats were individually housed and their body weight, food and water intake were monitored twice daily for the duration of the experiment. The groups consisted of: a) freely-fed 0h control, b) 24h fasted, c) 48h fasted, d) 48h fasted and 8h re-fed, e) 48h fasted and 24h re-fed, f) 48h fasted and 48h re-fed, and g) control group, freely-fed for 96h (n = 5 for each group) for juvenile and adult rats. At each time point, animals were sacrificed and liver and blood was collected for analysis. Compared to adult rats, juvenile rats had significantly lower baseline hepatic gene expression and serum levels of ALS (50% and 70% of 0h adult controls, respectively,  $p < 0.01$ ) and IGF-I (30% and 60% of 0h adult controls, respectively,  $p < 0.01$ ). After 24h of fasting hepatic ALS gene expression and serum levels were significantly decreased in juvenile rats (45% and 60% of juvenile 0h controls) and to a lesser extent in adult rats (74% and 67% of adult 0h controls), persisting until 8h of re-feeding. However, following 24h of re-feeding serum ALS levels rose to control values in juvenile rats but not in adult rats. Similarly, hepatic IGF-I gene expression was significantly suppressed after 24h fasting and persisted until 8h of re-feeding in both groups. In contrast, while significantly suppressed until 8h of re-feeding in adult rats, serum IGF-I levels in juvenile rats were suppressed within 48h of fasting and returned to normal values after only 8h of re-feeding. There was significant suppression of hepatic GH receptor (GHR) gene expression after 24-48h of fasting and 8h of re-feeding in juvenile (40-50% of 0h juvenile controls,  $p < 0.0002$ ) and to a lesser extent in adult rats (60-70% of 0h adult controls,  $p < 0.002$ ), and only after 24h of re-feeding did levels return to control values in both groups. This suppression, in part, is mimicked by decreased GH binding to liver microsomal membranes and hepatic gene expression of signal transducer and activator of transcription (STAT)-5b and Janus Kinase (JAK)-2. In contrast, fasting and re-feeding did not alter gene expression of the suppressor of cytokine signalling (SOCS)-3 or cytokine inducible SH2 sequence (CIS) proteins which remained at 0h control levels. ALS infusion experiments indicated that differences in serum levels of ALS and IGF-I in response to short-term fasting and re-feeding between juvenile and adult rats are not due to different ALS clearance rates which were found to be identical in both groups. The mechanism for regulation of ALS and IGF-I during short-term fasting involves regulation at GHR level, but the age-dependent response to re-feeding may in part be due to differential activation of the GH signalling pathway.  
(Supported by NHMRC, Australia)

## RELATIONSHIPS BETWEEN HEPATIC EXPRESSION OF INSULIN-LIKE GROWTH FACTOR-I (IGF-I) AND THE IGF ENDOCRINE SYSTEM ARE AFFECTED BY NUTRITION AND PREGNANCY

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Pregnancy increases the concentration of IGF-I in blood in humans and guinea pigs suggesting that IGF-I has endocrine actions in the mother. The aim of the present study was to determine the source of endocrine IGF-I in pregnancy in guinea pigs. Nulliparous 3 to 4 mo old female guinea pigs (n=28) were allocated to four treatments. Pregnant animals were either fed *ad libitum* or fed 30% less than this for 28 d before mating and for the first 30 d of pregnancy, then 10% less than *ad libitum* for the next 30 d (term is 65 d). Feed was similarly provided (*ad libitum* or restricted) to nonpregnant guinea pigs for 88 d. Animals were euthanased to obtain tissues and blood. IGF-I and -II were measured by specific RIAs after size exclusion hplc at pH 2.5. IGF-BP-3 was measured by densitometric analysis of Western ligand blots. IGF-I and  $\beta$ -actin mRNA were measured in liver RNA by RTPCR digoxigenin ELISA. Relative abundance of IGF-I mRNA was expressed as the ratio of IGF-I/ $\beta$ -actin RTPCR products. When data was combined from all treatments, relative abundance of IGF-I mRNA in liver (n=28) was strongly correlated with plasma concentrations of IGF-I [ $r = +0.71$ ,  $p < 0.0001$ ], IGF-II [ $r = +0.66$ ,  $p < 0.0001$ ], IGF-binding protein-3 [ $r = +0.56$ ,  $p < 0.002$ ], body weight [ $r = +0.68$ ,  $p < 0.0001$ ] and weights of spleen [ $r = +0.60$ ,  $p < 0.001$ ] and uterus [ $r = +0.71$ ,  $p < 0.0001$ ]. In pregnant animals overall (n=15), the ratio of IGF-I mRNA/ $\beta$ -actin in liver was similarly correlated with plasma levels of IGF-I [ $p < 0.01$ ], IGF-II [ $p = 0.001$ ] and IGF-binding protein-3 [ $p < 0.01$ ] as well as with body weight [ $p = 0.01$ ] and weights of spleen [ $p = 0.001$ ], thymus [ $p < 0.01$ ] and gravid uterus [ $p = 0.01$ ]. None of these relationships were statistically significant in nonpregnant guinea pigs [n=13, all  $r < +0.53$ ]. In all animals fed *ad libitum* (n=11), relative abundance of hepatic IGF-I mRNA was positively correlated with plasma IGF-I [ $r = +0.62$ ,  $p < 0.05$ ] but was not significantly related to any other measurements. In all feed restricted guinea pigs (n=17), hepatic expression of IGF-I was positively correlated with body weight [ $r = +0.54$ ,  $p = 0.02$ ] and negatively correlated with thymus weight [ $r = -0.49$ ,  $p < 0.05$ ]. The relationship between relative abundance of hepatic IGF-I mRNA and plasma concentration of IGF-I indicates that liver is the major source of endocrine IGF-I in guinea pig, as is known in rodents and is thought to be the case in humans. We propose that hepatic IGF-I production has anabolic actions on maternal somatic and immune tissues, as well as the conceptus, during pregnancy.

## PHOSPHORYLATION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-5 AND ITS ROLE IN COMPLEX FORMATION WITH THE IGFS AND THE ACID-LABILE SUBUNIT

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The insulin-like growth factors (IGF-I and -II) circulate in high concentrations and it is believed a family of binding proteins (IGFBPs) regulate their bioavailability by transporting the IGFs, prolonging their half lives and regulating their transcapillary passage to target tissues. Of the six structurally related IGFBPs, only IGFBP-3 and, more recently, IGFBP-5 have been shown to circulate as ternary complexes with an IGF and a liver derived glycoprotein, the acid-labile subunit (ALS). There is potential for up to 10% of circulating IGFs to exist in the ternary form bound to IGFBP-5, which is sufficient to exert major glucoregulatory or mitogenic activity. Within the cellular environment, the ability of IGFBP-5 to modulate the effects of the IGFs is well characterised with the potential to either inhibit or potentiate IGF signalling through the type 1 IGF receptor. It has also been previously demonstrated that ALS has a potentiating effect on IGFBP-5 inhibition of IGF-I induced cell proliferation, possibly via formation of the ternary complex. Phosphorylation has been proposed as a regulatory factor affecting ternary complex formation and hence may influence the bioavailability of the IGFs. The aims of this study were to investigate whether IGFBP-5 is secreted as a phosphoprotein and to study the effect phosphorylation may have on its ligand binding. The human breast cancer cell line, T47D, was shown to secrete high amounts of IGFBP-2, -4 and -5 and subsequently this cell line was used as an *in vivo* model for metabolic labelling. Cells were made serum-free and phosphate-free before the addition of  $^{32}\text{P}$ -orthophosphate. Conditioned media was collected at various time points and IGFBPs precipitated out using IGF-I conjugated beads, and analysed by SDS-PAGE and autoradiography. Specific incorporation of  $^{32}\text{P}$  into newly synthesised IGFBP-5 was observed from 4 hours and maximally by 12 hours but IGFBP-2 and -4 were not phosphorylated. Since IGFBP-5 contains a number of consensus phosphorylation sites, recombinant human IGFBP-5 (rhIGFBP-5) was incubated with different kinases in the presence of  $^{32}\text{P}$ -ATP. Analysis by SDS-PAGE and autoradiography revealed specific phosphorylation by PKA, CKII, PKC and Cdc2, but not by MAPK. To investigate the ligand binding activity of phospho-IGFBP-5, non-radiolabelled PKA or CKII phosphorylated rhIGFBP-5 was prepared, purified by reverse-phase HPLC and quantitated by a specific radioimmunoassay (RIA). As controls, mock reactions where each kinase was omitted were also prepared. The *in vitro* phosphorylation of rhIGFBP-5 did not alter its immunoreactivity with the specific Ab used in the quantitative RIA. Ligand binding was assessed by incubating phosphorylated rhIGFBP-5 with either  $^{125}\text{I}$ -IGF-I or  $^{125}\text{I}$ -IGF-II and resulting binary complexes immunoprecipitated with  $\alpha$ -IGFBP-5 Ab, or  $^{125}\text{I}$ -IGF-I and ALS and resulting ternary complexes immunoprecipitated with  $\alpha$ -ALS Ab. Phosphorylation by PKA or CKII did not change the ability of rhIGFBP-5 to bind IGF-I, IGF-II or ALS in the presence of IGF-I tracer as compared to the mock preparations. This study confirms that IGFBP-5 is secreted as a phosphoprotein. However, *in vitro* phosphorylation of IGFBP-5 by PKA or CKII does not modulate IGF and ALS binding activity. Although the hypothesis that phosphorylation modulates IGFBP-5 binding and hence affects IGF bioavailability is as yet unfounded, it may possibly do so indirectly by regulating IGFBP-5 binding to other ligands and/or cell surfaces or by altering its susceptibility to proteolytic cleavage.

## GENDER DIFFERENCES IN PROGRAMMING OF ADOLESCENT IGF-I SENSITIVITY BY PERINATAL GROWTH.

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Small size at birth, and failure of subsequent neonatal catch-up growth, are both associated with reduced stature and poor metabolic control in the adult. This may be in part due to impaired IGF-I secretion or sensitivity, which has been observed in the neonate and child following poor prenatal growth<sup>1-3</sup>. If such impairments persist, they may limit adolescent growth and also contribute to the reduced height and poor metabolic homeostasis observed following IUGR. The effects of IUGR on the IGF-I axis in later life are poorly defined however. We therefore determined the effects of placental restriction of fetal growth (PR) on *in vivo* sensitivity to IGF-I in adolescent sheep, and evaluated the influences of size at birth and neonatal growth rate on subsequent IGF-I sensitivity.

PR was induced by removal of the majority of endometrial caruncles prior to mating. Size and weight were measured at birth and at regular intervals thereafter. Fractional growth rates (FGR) from 0-45 days were calculated as absolute growth rate divided by size at birth. Indwelling arterial and venous catheters were inserted at least 2 weeks before experiments. The IGF-I sensitivity of glucose metabolism was measured by hyper-IGF-I euglycaemic clamp in fasted sheep at  $136 \pm 1$  days of age (n=15 control, n=13 PR sheep). Blood glucose was measured in blood taken at five minute intervals for 15 min prior to, and during each clamp. IGF-I (3 micrograms/kg body weight/min) was infused for 130 min. Glucose was infused concomitantly from 25 min after the start of the IGF-I infusion to restore and maintain euglycaemia. IGF-I sensitivity was calculated as the steady state glucose infusion rate (70-130 min). The effects of PR and gender were tested by 2-way ANOVA. Relationships between variables were analysed by linear regression.

PR and gender did not alter IGF-I sensitivity in adolescent sheep. In adolescent males, IGF-I sensitivity in adolescence was positively related to weight ( $r=-0.63$ ,  $P=0.020$ ,  $n=13$ ) and crown-rump length ( $r=0.63$ ,  $P=0.022$ ,  $n=13$ ) at birth, and was negatively related to neonatal FGR in terms of weight ( $r=-0.57$ ,  $P=0.068$ ,  $n=11$ ) or shoulder height ( $r=-0.62$ ,  $P=0.041$ ,  $n=11$ ). Conversely, IGF-I sensitivity in adolescent females was not related to weight or crown-rump length at birth ( $P>0.1$ ), and was negatively related to length of long bones at birth (metatarsus length:  $r=-0.58$ ,  $P=0.029$ ,  $n=14$ ). Also in contrast to males, IGF-I sensitivity in adolescent females was positively related to neonatal FGR in terms of shoulder height ( $r=0.57$ ,  $P=0.052$ ,  $n=12$ ), and unrelated to neonatal FGR in terms of weight ( $P>0.8$ ).

Thus, poor prenatal growth and rapid neonatal growth are associated with impaired IGF-I sensitivity in adolescent males, but with enhanced IGF-I sensitivity in adolescent females. The extent to which this represents gender differences in perinatal programming of IGF-I sensitivity or in maturation rate following IUGR remains to be determined.

## THE EFFECT OF SUPPRESSION OF CORTISOL SYNTHESIS ON FETAL ARTERIAL BLOOD PRESSURE AND GROWTH IN THE LATE GESTATION FETAL SHEEP.

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Cortisol plays an important role in the development of the fetal cardiovascular system and in fetal growth. In the late gestation fetal sheep a prepartum rise in cortisol occurs concomitantly with an increase in fetal arterial blood pressure and a decrease in fetal growth. The aims of this study were to determine the effect of suppression of endogenous cortisol synthesis on arterial BP between 125d and 140d gestation and on growth in the fetal sheep.

Surgery was performed at 119-120d gestation (n=14) to insert fetal and maternal catheters for infusion studies, blood pressure measurements and collection of blood samples. Metyrapone (0.9M/h; a competitive inhibitor of 11 $\beta$  hydroxylase; n=6) or vehicle, (0.6M tartaric acid; n=8), were infused into the fetal jugular vein between 125d and 140d. Mean arterial blood pressure was measured continuously for 8h at 126-127 d and at 137-140d. At 140d a post mortem was performed where fetal body and organ (adrenal and kidney) weights were measured.

Fetal plasma concentrations of cortisol were reduced in the metyrapone infused fetuses at 127 d ( $4.2 \pm 1.5$  nmol/L) and 139 d ( $18.1 \pm 3.6$  nmol/L) when compared to the vehicle infused animals ( $6.3 \pm 3.3$  nmol/L; 127d) and ( $35.8 \pm 22.5$  nmol/L; 139d) respectively. The mean arterial BP in the metyrapone infused fetuses was significantly lower ( $p < 0.05$ ) at 126-127d ( $43 \pm 2.0$  mmHg) and at 137-139d ( $49 \pm 0.9$  mmHg) when compared to vehicle infused animals ( $47 \pm 2.1$  mmHg; 126-127d) and ( $53 \pm 3$  mmHg; 137-139d), respectively. Mean arterial blood pressure was higher, however, in both metyrapone and control animals at 137-140d, when compared to 126-127d. At 140d, adrenal weights were significantly increased ( $p < 0.005$ ) in the metyrapone infused fetuses ( $0.79 \pm 0.07$ g) when compared to the vehicle infused fetuses ( $0.41 \pm 0.04$ g). Kidney weights were also significantly increased ( $p < 0.05$ ) in the metyrapone infused fetuses ( $35.3 \pm 2.1$  g) when compared to the vehicle infused fetuses ( $24.4 \pm 2.2$  g). This study provides evidence that endogenous fetal cortisol plays a role in the maintenance of fetal arterial blood pressure during late gestation and is important in the regulation of growth of the fetal adrenals and kidneys.

## PROSTAGLANDIN H SYNTHASE TYPE-2 (PGHS-2) AND PROSTAGLANDIN DEHYDROGENASE TYPE-1 (PGDH) EXPRESSION IN GESTATIONAL TISSUES OF THE GUINEA PIG.

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Elevated intrauterine prostaglandin (PG) levels induce parturition by stimulating myometrial contractions, cervical softening and membrane rupture. The concentration of bioactive PGs in the uterus depends on the activity of prostaglandin biosynthetic and metabolic pathways in the gestational tissues (amnion, chorion, decidua, placenta). The key rate-limiting enzymes of PG biosynthesis and metabolism are prostaglandin endoperoxide H synthase type-2 (PGHS-2) and 15-hydroxyprostaglandin dehydrogenase type-1 (PGDH), respectively. In human pregnancy, PGHS-2 activity increases and PGDH activity decreases in the uterus before and during term and preterm labour. This results in elevated bioactive PG levels, initiating and maintaining labour. The endocrine/paracrine mechanisms responsible for these changes in enzyme expression are largely unknown. Our aim is to explore the intrauterine regulation of PGHS-2 and PGDH using an animal model of human pregnancy where the experimental manipulation of hormonal systems may be performed. We use the guinea pig for this purpose, because its metabolic and endocrine characteristics show important similarities to humans at late gestation. Little information exists, however, regarding the expression of PGHS-2 and PGDH in guinea pig gestational tissues. In the present study, we determined the levels of PGHS-2 and PGDH mRNAs in guinea pig gestational tissues as a first step towards establishing this animal model.

**METHODS:** Myo-endometrium (n=4), amnion (n=12), chorion (n=11) and placenta (n=10) were obtained from pregnant guinea pigs during the last third of gestation (44-69 days; term = 58-72 days). Total RNA was extracted, column-purified and treated with DNase. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to determine the levels of mRNAs encoding PGHS-2 and PGDH relative to GAPDH mRNA. Results were analysed by one-way ANOVA followed by Tukey's post hoc test and Student's t-test (paired), as appropriate.

**RESULTS:** We have developed real time quantitative RT-PCR procedures to measure the abundance of guinea pig-specific PGHS-2, PGDH and GAPDH mRNAs. These mRNAs were detected in all guinea pig gestational tissues analysed. PGHS-2 mRNA was significantly ( $p < 0.05$ ) more abundant in the placenta than in the other tissues. The level of PGDH mRNA was higher ( $p < 0.05$ ) in the chorion than in the amnion, placenta and myo-endometrium. PGHS-2 gene activity, as reflected by mRNA levels, was significantly higher than PGDH gene activity in the amnion and placenta ( $p < 0.05$ ). In contrast, PGDH mRNA level in the chorion was more than five-fold greater than that of PGHS-2 mRNA ( $p < 0.05$ ).

**CONCLUSIONS:** These preliminary data indicate that the gestational tissue-specific patterns of PGHS-2 and PGDH expression are similar in guinea pigs and humans. Thus, the placenta and the amnion are major intrauterine sites of PG production, while PG metabolism takes place predominantly in the chorion membrane in both species. The results support the suitability of the guinea pig as an animal model of human pregnancy, and provide the basis for further studies to determine the hormonal regulation of intrauterine PG biosynthetic and metabolic pathways at parturition. Supported by NH&MRC and RMC University of Newcastle.

## CHARACTERISATION OF STANNIOCALCIN-1 KNOCKOUT MICE

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Stanniocalcin (STC) is a hormone previously considered to be present only in bony fish where it is secreted by the corpuscles of Stannius, endocrine organs involved in calcium and phosphate homeostasis (1). Its site of actions are the gills, the kidney and the gut. We and others (2-4) identified cDNAs encoding human and mouse stanniocalcin (STC1). Instead of being expressed in a single organ like the fish counterpart, mammalian STC1 mRNA has been identified in a wide variety of tissues, including the kidney, prostate, thyroid, bone and ovary. Due to its expression in multiple organs during development and in adult mice, many functions have been suggested for STC1, including renal calcium and phosphate handling, response to hyperosmolarity, protection against cerebral ischemia, a role in angiogenesis, mediation of inflammation and wound healing, and also a role in embryologic development, especially of the musculoskeletal system.

In order to determine the physiological functions of STC1, we generated STC1-null mice by gene targeting. We found that STC1-null mice are capable of undergoing apparently normal development. Further investigations of the STC1-knockout phenotype are currently being carried out.

## IS THERE A ROLE FOR CRH IN THE METAMORPHOSIS OF AUSTRALIAN FROGS?

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Metamorphic processes that allow a tadpole to develop into a frog are instigated by the direct action of thyroid hormone within individual tissues. Thyroid hormone is produced in response to stimulation by thyroid stimulating hormone (TSH), which in turn is produced in response to thyrotropin-releasing hormone in vertebrates including adult frogs. However, this is not the case in larval (metamorphosing) frogs. Studies on a limited number of Northern Hemisphere frog and toad species suggest that the stress hormone corticotropin-releasing hormone (CRH) stimulates TSH production and regulates thyroid hormone during metamorphosis (Denver, 1997). In these studies an early rise in CRH coincided with early metamorphosis of desert-dwelling toads in response to stressful environments such as pond desiccation. Injection of CRH into Northern American toads *Scaphiopus hammondi*, *Rana perezi* and *Bufo arenum* induced an early metamorphosis, whereas CRH receptor antagonists and anti-CRH antibodies delayed metamorphosis.

This study examines the involvement of CRH in the metamorphosis of two Australian species from one of the 2 major frog families endemic to Australia, both of which have evolved independently of Northern American species for at least 175 million years. Change in brain CRH was measured throughout metamorphosis by dissecting out the brain and extracting the CRH peptide using a water extraction method, then measuring the CRH peptide by radioimmunoassay (RIA). There were 4 tadpoles harvested at each of the 6 stages investigated. These were pooled into 2 groups of 2 brains for CRH detection, with each pool being assayed in duplicate. Results were expressed as pg CRH per mm body length as brains were too small to weigh during most of metamorphosis.

CRH was detected throughout metamorphosis in Red Eyed Green Tree Frog (*Litoria chloris*) tadpoles, with a rise during the later, most obvious stages of forelimb emergence and tail resorption. This is consistent with the hypothesis that CRH is involved in the metamorphosis of frogs and toads. The effect of exogenous CRH on metamorphosis was also determined by giving 3 injections of CRH into the peritoneal cavity of pre-metamorphic tadpoles over 5 days, at concentrations of 0, 2 or 5 µg per gram of body mass. However, injections of CRH did not result in any significant difference in the timing of development in the Green & Golden Bell Frog (*Litoria aurea*), suggesting that CRH has no inductive effect on metamorphosis when given at these doses at this stage of development.

## THE EXPRESSION AND ACTION OF GHRELIN AND THE GROWTH HORMONE SECRETAGOGUE RECEPTOR IN PROSTATE CANCER CELL LINES.

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Synthetic growth hormone secretagogues (GHSs) are potent inducers of growth hormone (GH) secretion in humans, as demonstrated by both *in vivo* and *in vitro* studies. These peptide and non-peptide molecules were initially developed for clinical use in disease states such as growth hormone deficiency. The use of GHS treatment in the aged, where there is a decline in GH secretion, has been suggested to restore pulsatile GH secretion, possibly reversing the age-related changes in body composition and mental facility. GHS action is mediated by the GHS receptor (GHS-R), of which there are two identified subtypes, 1a and 1b. Ghrelin, the endogenous ligand for the GHS-R has only recently been isolated and characterised. Ghrelin and the GHS-R are now acknowledged as new components of the growth hormone (GH) axis, thereby contributing to the release of GH in the body. An increasing body of research has implicated a role for some components of the GH axis in the development and maintenance of prostate cancer. For example, antagonists of growth hormone releasing hormone (GHRH), the traditionally recognised growth hormone secretagogue, inhibit the growth of prostate tumours *in vivo* and prostate cancer cells *in vitro*. Our research is an investigation of the expression of ghrelin and the GHS-R in prostate cancer cells and prostate tumour tissue. The effect of exogenous ghrelin on prostate cancer cells was also examined. GHS-R 1a and 1b mRNA isoforms and ghrelin mRNA expression were detected by reverse transcriptase PCR (RT-PCR) in the androgen-dependent ALVA-41 and LNCaP and in the androgen-independent DU145 and PC-3 prostate cancer cell lines. Normal prostate cDNA expresses ghrelin and the GHS-R 1a isoform mRNA but not the 1b isoform. Using immunohistochemical methods, we demonstrated that all of the prostate cancer cell lines studied express the GHS-R 1a isoform and ghrelin at the protein level. Immunoreactivity for ghrelin was also detected in normal and malignant prostate epithelium of paraffin-embedded prostate tumour tissue with more intense staining noted in the cancerous glands compared with the normal glands, suggesting increased ghrelin expression in malignant prostate glands. In cell culture experiments, incubation of PC-3 cells with ghrelin increased their proliferation to levels 33% above untreated controls, implying a potential tumour-promoting role for ghrelin in the prostate. This research is the first demonstration of the co-expression of the GHS-R and its natural agonist, ghrelin, in prostate cancer cells. In addition, our functional studies provide evidence that a previously unrecognised autocrine/paracrine pathway involving ghrelin, which is capable of stimulating growth, exists in prostate cancer. In light of the data presented, caution is indicated regarding the use of GHSs, which mimic ghrelin, in medical and non-medical settings, particularly in the aging male.

## EXPRESSION AND REGULATION OF THE NEW PROSTATIC KALLIKREIN, *KLK4*, IN PROSTATE CANCER CELL LINES

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Tissue kallikreins (*KLKs*) belong to a multigene family of serine proteases. Two members of the human kallikrein gene family which are highly expressed in the prostate, prostate specific antigen (PSA) and K2, have been implicated in facilitating human prostate cancer cell proliferation and invasion. PSA is also the most useful serum marker for cancer detection and monitoring. Recently, the human kallikrein gene family has been extended to fifteen genes<sup>1</sup>. One of these genes, *KLK4*, which is also highly expressed in the prostate<sup>2</sup>. The mouse orthologue of *KLK4* has been implicated in bone matrix degradation suggesting that *KLK4* may play an important role in prostate cancer invasion and metastasis. Androgens and thyroid hormone ( $T_3$ ) are known to differentially regulate the expression of PSA and *KLK2* in the LNCaP prostate cancer cell lines<sup>3</sup>. Thus, the aim of this study was to examine the expression of *KLK4* in the androgen dependent prostate cancer cell lines, LNCaP and ALVA-41, and the androgen independent lines, DU-145 and PC-3, as well as the potential regulation of *KLK4* by dihydrotestosterone (DHT) and triiodothyronine ( $T_3$ ).

Using RT-PCR and subsequent Southern blot and DNA sequencing analysis, *KLK4* expression was confirmed in LNCaP cells, and also detected in DU145 and PC-3 cancer cell lines. This is in sharp contrast to PSA, which is only expressed in LNCaP cells. Using quantitative RT-PCR (Idaho Lightcycler), *KLK4* regulation by DHT in LNCaP cells was examined and shown to be up-regulated by 10 nM DHT (1.7 fold increase) after 48 h stimulation. K4 protein levels, as detected by Western blot analysis, also increased with increasing concentrations of DHT treatment (0, 0.1, 1.0, 10 nM DHT) after 48 hours. Densitometry analysis showed a 1.8 fold increase in K4 protein levels between the 0 and 10nM DHT treatment groups. A similar pattern was observed for PSA expression and secretion as expected. Although  $T_3$  alone appeared to decrease *KLK4* levels, *KLK4* mRNA transcript and K4 protein levels were up-regulated by a combination of 10nM DHT with 100nM  $T_3$  after 48 h stimulation. Although these findings need to be confirmed, these studies suggest that like PSA, but not like *KLK2*, *KLK4* expression is regulated by both DHT and  $T_3$  in LNCaP cells. The expression of *KLK4* in both androgen-dependent and -independent prostate cancer cell lines suggests a potential role of *KLK4* in prostate cancer progression. Further regulatory and functional studies are being performed in order to more precisely understand the role of this enzyme in prostate tumourigenesis.

## CHARACTERISATION OF PROSTATE-SPECIFIC ANTIGEN (PSA) OVER-EXPRESSION IN THE HUMAN PROSTATE CANCER CELL LINES, ALVA-41 AND PC-3

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Prostate-specific antigen (PSA), a member of the kallikrein family of serine proteases, is the current biological marker for prostate cancer used both for tumour detection and monitoring recurrence. PSA acts as a seminal liquefaction enzyme reproductively, but recent evidence has indicated that it may also be involved in prostate cancer progression either as a tumour promoter by cleaving the IGF/IGFBP-3 complex to release bioactive IGF-1<sup>1</sup>, or alternatively, as a tumour suppressor due to apoptotic and anti-angiogenic activity<sup>2,3</sup>. However, its specific function in prostate cancer is still unclear. Hence, the aim of this project is to determine the functional consequences of PSA over-expression in two prostate cancer cell lines, ALVA-41 and PC-3.

Briefly, two separate constructs were made using the mammalian expression vector, pcDNA3: (1) PSA-short, incorporating the PSA coding region only, and (2) PSA-long, incorporating the PSA coding region and a portion (530bp) of the 3' untranslated region of the PSA mRNA transcript. These constructs were stably transfected into ALVA-41 and PC-3 cells using the calcium phosphate co-precipitation method or cationic lipid reagent-mediated transfection system, respectively. These cell lines were chosen because neither endogenously expresses PSA at the mRNA or protein levels. Between 5 and 12 clones from both cell lines were made for each construct, and their PSA-expressing ability was confirmed by immunocytochemistry, RT-PCR and PSA immunoassay. Interestingly, PC-3:PSA clones, in particular, appear smaller and more fibroblast-like, when compared to the larger, more rounded parent cells. The reason for this is unknown. To determine the effect of PSA over-expression on these cell lines, a variety of functional parameters have been/will be measured. Initial studies indicate that ALVA-41:PSA and PC-3:PSA clones decrease cell growth when using a colorimetric MTT (tetrazolium) proliferation assay, however, vector only clones also showed a decrease in growth when compared to the native untransfected parent cells. Additionally, when conditioned medium from ALVA-41:PSA clones was taken and incubated with parent cells, there was also a corresponding decrease in cell proliferation. To determine any differences in the invasive ability of ALVA-41 parent cells and the ALVA-41:PSA cells, invasion assays were performed in Boyden chambers containing Matrigel, a synthetic extracellular matrix. Results showed that PSA expression did not alter the invasive capacity of these cells. Further studies using PC-3:PSA in this system are on-going.

In conclusion, although yet to be confirmed, these results suggest that PSA over-expression decreases the proliferative activity of prostate cancer cell lines but does not alter the invasive potential of ALVA-41 cells. Studies are on-going to further investigate these findings and other potential functional changes in these cell lines.

## CHROMOGRANIN A - A MARKER OF NEUROENDOCRINE TUMOUR METASTASIS IN MULTIPLE ENDOCRINE NEOPLASIA TYPE 1 (MEN 1)

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**Background** Chromogranin A (CgA), a water-soluble acidic glycoprotein present in the densecore vesicles of neuroendocrine and neuronal tissues, is co-secreted with the hormone contents of secretory granules. These characteristics have made CgA a valuable immunohistochemical and serum marker for neuroendocrine tumour, exhibiting high specificity, and direct correlation with tumour volume. Multiple Endocrine Neoplasia Type 1 (MEN 1) is an autosomal dominant syndrome associated with benign and malignant neuroendocrine neoplasia. Malignant gastroenteropancreatic (GEP) neoplasms are a major cause of death in MEN 1. Early recognition of those patients with occult GEP tumours at high risk of malignant transformation / metastasis may permit pre-emptive intervention. However, the early identification of high risk patients has thus far proved difficult.

**Methods** Data for 21 MEN 1 patients aged > 30 years followed for up to seven years (2 – 7 years) with regular assessments of serum CgA were reviewed. The utility of serum CgA as a marker of patients at high risk for developing GEP metastases was assessed using a cross-sectional and longitudinal study methodology.

**Results** At baseline, three patients (21%) had radiological evidence of metastatic GEP malignancy, seven satisfied biochemical criteria for gastrinoma (33%), and eleven (52%) had small (<3cm) benign "non-functioning" pancreatic adenomata. CgA was elevated by greater than six-fold in: all (100%) patients with metastases; two patients with gastrinoma (29%); and none of those patients with small benign non-functioning pancreatic adenoma. During follow-up, two patients (10%) developed new GEP metastatic malignancy, which was associated with CgA increasing from normal to elevated levels.

**Conclusion** CgA is both a useful marker for both GEP tumour extent, as well as a biochemical indicator of the transition to metastatic malignant GEP neoplasia. It is not however clear if the increase in CgA precedes metastatic spread.



## THE EFFECT OF STRESS AND/OR COUNSELLING ON REMISSION RATES GRAVES' DISEASE.

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A relationship between stressful life events and the onset of Graves' disease (GD) has long been suspected, and although studies have been inconsistent, more recent publications have indicated a strong association [1,2]. We describe here a pilot study, evaluating the hypothesis that addressing patients' prevailing stressors, may improve the likelihood of remission in GD.

GD was considered confirmed when T<sub>4</sub> and/or T<sub>3</sub> levels were elevated above the reference range, TSH was suppressed, and a Technetium scan showed diffuse uptake. Consecutive patients with GD (n = 18) were assessed subjectively from the history, as to the presence and level of stress prior to the onset of symptoms. They were assigned to three broad groups; low/no stress, moderate stress and high stress. The latter assessment was subsequently validated against a published life-events scale [3], with the three groups having average scores of 25, 80, and 105 respectively. High stress life-events, related in the main to severe marital disturbance and illness in, or separation from, children. All patients were treated with titrated carbimazole for 12-18 months, and received simple reassurance, acknowledgment and support, if stress was an issue. Patients were prospectively studied, with the relevant outcome being the attainment of remission at the end of treatment. Remission was defined as the maintenance of normal T<sub>4</sub> and T<sub>3</sub> levels, for at least 6 months, after carbimazole discontinuation.

We found a significant relationship (p<0.01) between the degree of stress and the likelihood of remission. Thus all patients with high or moderate stress (n=12) achieved remission as compared to only 1 of 6 patients in the no/low stress category.

Addressing stressful issues therefore may improve the rate of remission in GD. Alternatively, GD associated with a precipitating life-event, may have a favourable prognosis. The results justify a larger study.

## REDUCED BONE SIZE AND VOLUMETRIC BONE MINERAL DENSITY IN MEN RECEIVING ORAL CORTICOSTEROID THERAPY

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There is evidence to suggest that reduced bone formation is primarily responsible for corticosteroid-related bone deficits. We hypothesised that men receiving corticosteroid therapy (CST) for chronic illnesses would have reductions in both bone mineral content (BMC) and bone size compared with healthy control subjects. A total of 142 men aged 21 to 88 years (mean 62.6 yrs) who were treated with prednisolone for various chronic inflammatory illnesses were studied. The patients received an average of 14.1 mg of CST daily (range, 1-75 mg) for 7.4 yrs (range, 0.08 to 40 yrs). Controls comprised of 395 healthy men aged 17 to 91 years. Bone size and volumetric bone density (vBMD) at the third lumbar vertebra and femoral neck were measured as previously described (1). Results are expressed as Z-scores i.e. the number of standard deviation (SD) scores above or below the age-matched normal mean. Patients who received CST had reduced vertebral width ( $-0.38 \pm 0.08$  SD,  $p < 0.001$ ), height ( $-0.22 \pm 0.08$  SD,  $p < 0.01$ ), volume ( $-0.44 \pm 0.1$  SD,  $p < 0.001$ ), BMC ( $-0.75 \pm 0.09$  SD,  $p < 0.001$ ) and vBMD ( $-0.64 \pm 0.08$ ,  $p < 0.001$ ). Reductions were also seen in femoral neck width ( $-0.21 \pm 0.07$  SD,  $P < 0.01$ ), volume ( $-0.26 \pm 0.09$ ,  $p < 0.01$ ), BMC ( $-0.72 \pm 0.10$  SD,  $p < 0.001$ ), and vBMD ( $-0.57 \pm 0.08$  SD  $p < 0.001$ ). The deficits in bone volume exaggerated the deficits in BMC by 50% at the vertebrae and by 28% at the femoral neck. After adjustment for age, there was no relationship between bone width or vBMD and cumulative dose of CST ( $r = 0.02$  to  $0.13$ , all NS). These observed reductions in bone size may possibly be a result of reduced periosteal bone formation secondary to CST, the chronic illness or a combination of both processes. In summary, men who have received CST are at increased risk for vertebral and hip fractures because of the smaller bone size and reduced vBMD.

## “STRESS HYPERGLYCEMIA”: COMMON IN ACUTE CORONARY SYNDROMES

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Varying degrees of hyperglycemia is frequently observed in individuals without prior history of diabetes mellitus (DM), following acute coronary syndromes. Unfortunately, this is usually labeled as “stress hyperglycemia” and often ignored in an acute hospital setting for various reasons, one of which is an unfounded belief that it is a self-resolving benign condition. The aims of this study were to determine:

the prevalence of undiagnosed DM and “stress hyperglycemia” in patients admitted to Coronary Care Unit (CCU) with acute coronary syndromes,  
the factors (both clinical and laboratory) which best predict the presence of previously undiagnosed hyperglycemia.

In this prospective study, we measured fasting serum glucose and simultaneous HbA1c of consecutive patients admitted to CCU at our hospital with acute coronary syndromes between June 2000 and December 2000. Fasting hyperglycemia was defined as  $>5.5$  mmol/L. Details of individual patient’s medical and family history as well as admission cardiac diagnosis were recorded. Review of symptoms suggestive of DM was also undertaken. In addition, waist circumference (WC) measurements were recorded in all mobile patients.

Total of 327 patients were assessed during this study period (342 single admissions, in which 15 did not have glucose measurement). 26.2% (N=90/342) of patients admitted with coronary artery disease had prior diagnosis of DM. Of patients with coronary artery disease and without prior history of DM, 68% (N=161/237) had fasting serum glucose of  $>5.5$  mmol/L. Additional 3% (N=7/237) were newly diagnosed with Type 2 DM while in hospital, based on simultaneous measurements of fasting serum glucose and HbA1c. Patients with acute myocardial infarction (AMI) had fasting serum glucose level 0.7 mmol/L higher than those who presented with unstable angina pectoralis (UAP), when adjusted for the day of blood sampling. Age, sex, family history of diabetes and WC did not contribute to variation in glucose levels. Multivariate regression analysis showed that only acute cardiac diagnosis and presence of polyuria and visual changes predicted the presence of fasting hyperglycemia, adjusted for the day of blood sampling.

Elevated blood glucose level is highly prevalent in patients with coronary artery disease at the time of an acute presentation. A close follow-up of these individuals is essential to delineate their true state of glucose tolerance upon resolution of an acute stress.

## TREATMENT WITH AQUEOUS EXTRACTS OF VIJAYASAR (*PTEROCARPUS MARSUPIUM*) INCREASES INSULIN RESPONSIVENESS TO ELVEVATED BLOOD GLUCOSE IN SHEEP

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Although various extracts of the heartwood from the tree *Pterocarpus marsupium* (Vijayasar) has been used for centuries by Indian folk medicine practitioners as a treatment for diabetes there have been few comprehensive studies of its physiological effects (1). The aim of this study was to investigate the effect of vijayasar on glucose metabolism in a non-diabetic model. Although sheep are ruminants, the metabolic and endocrine pathways involved in glucose metabolism are similar to humans, and sheep are a more tractable model for these kinds of studies than are rodents. An aqueous extract of vijayasar was made by soaking a piece of wood overnight in normal saline and then filtering with a 0.22  $\mu$ m filter prior to use. To investigate the effect of this extract on basal glucose levels male Merino castrate sheep (wethers) were randomly divided into 2 groups (n=5) and one group of given an intravenous dose of vijayasar and the other group saline. Blood samples were taken hourly for 8 hours and then at 24 hours after the treatment. A second dose of vijayasar was given and a glucose tolerance test performed one hour later using an intravenous dose of glucose (2.5 mg/kg body weight) with blood samples taken at 15, 30, 45, 60, 120, 180 minutes post injection. The treated group received a dose of vijayasar every day for a further 3 days and then a glucose tolerance test was performed 8 hours after the last treatment.

In the first experiment no significant change in glucose was observed at any of the time points or between the vijayasar (58.2  $\pm$  2.0 mg/dl) and the saline (56.3  $\pm$  1.9 mg/dl) groups. Basal insulin levels were also not significantly different between the treated (13.3  $\pm$  2.9  $\mu$ U/ml) and saline (11.9  $\pm$  5.5  $\mu$ U/ml) groups. The first glucose tolerance test showed a slight but significant increase in the glucose plasma clearance rate in the vijayasar group (133.4  $\pm$  10.6 ml/min) compared to the controls (113.7  $\pm$  9.2 ml/min). Insulin levels at 15 minutes after glucose administration were significantly elevated in the treated group (166.7  $\pm$  27.6  $\mu$ U/ml) compared to the saline group (95.8  $\pm$  18.2  $\mu$ U/ml). The plasma clearance rate increased to 182.5  $\pm$  15.8 mls/kg in the vijayasar group compared to 106.0  $\pm$  23.5 in the saline group after the final treatment. Insulin was also significantly (P < 0.05) increased to 310.0  $\pm$  99.9  $\mu$ U/ml in the treated group compared with the saline group (189.5  $\mu$ U/ml).

These data show that the active ingredients in the extract significantly enhance the pancreatic  $\beta$  cell responsiveness to elevated blood glucose without any modulation of the basal levels of either insulin or glucose.

## INEQUALITY OF INTRACELLULAR CALCIUM RESPONSES TO ATP AND GLUCOSE IN THE RAT AND MOUSE PANCREATIC BETA-CELLS IN PRIMARY CULTURE

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Extracellular ATP has been demonstrated to stimulate insulin secretion from rat and human pancreatic islets, but inhibit insulin release from mouse pancreatic islets. The mechanism for such a species difference is unknown. Secretion of insulin is directly triggered by the elevation of intracellular free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ). Aiming to clarify the mechanism underlying the discrepancy between responses of beta-cells to ATP in rats or mice, we studied the alteration of  $[\text{Ca}^{2+}]_i$  in immunocytochemically identified beta-cells. Both mouse and rat islets were isolated by collagenase digestion and dispersed into single cells. Cells were cultured for 2-4 days and loaded with Fluo-3/AM on the experimental day. Levels of  $[\text{Ca}^{2+}]_i$  was measured using the Olympus confocal microscope and analysed using the software Fluoview-3. Under the low glucose conditions (2mM) in rat cells, ATP (20  $\mu\text{M}$ ) caused a rapid, large and transient increase in  $[\text{Ca}^{2+}]_i$  (first phase, a duration of  $80 \pm 10$  seconds with a fluorescent intensity increase to 203% of control), followed by a sustained plateau of increase in  $[\text{Ca}^{2+}]_i$  (second phase, with the fluorescent intensity higher than control level for more than 10 min). After the depletion of extracellular  $\text{Ca}^{2+}$  by EGTA (2 mM), the first phase increase in  $[\text{Ca}^{2+}]_i$  was not affected, but the second phase sustained increase in  $[\text{Ca}^{2+}]_i$  was abolished. In mouse cells, only the first transient increase in  $[\text{Ca}^{2+}]_i$  was observed (a duration of  $16 \pm 3$  seconds, with the fluorescent intensity increase to 436% of control), which was not eliminated by the depletion of extracellular  $\text{Ca}^{2+}$  with EGTA. After administration of glucose (9mM) in bath solution, both rat and mouse beta-cells showed an increase in  $[\text{Ca}^{2+}]_i$ , but the patterns of increase in  $[\text{Ca}^{2+}]_i$  were different between two species. The glucose-induced increase in  $[\text{Ca}^{2+}]_i$  was slow in rat beta-cells and went down to the control level within ten minutes without oscillation. In mouse beta-cells, the  $[\text{Ca}^{2+}]_i$  was increased by glucose quickly and maintained in a high level integrated by the  $[\text{Ca}^{2+}]_i$  oscillation. Under 9mM glucose condition, ATP (20  $\mu\text{M}$ ) induced the above mentioned two phases of increase of  $[\text{Ca}^{2+}]_i$  in rat beta-cells. It, however, did not induce significant increase in  $[\text{Ca}^{2+}]_i$  in mouse beta-cells. Both P2 receptor antagonist (Reactive Blue-2) and the depletion of intracellular  $\text{Ca}^{2+}$  stores by thapsigargin blocked the effect of ATP on both rat and mouse beta-cells. Based on above results, we conclude (1) ATP directly induces two phases of increase in  $[\text{Ca}^{2+}]_i$  in rat beta-cells but only one transient phase in mouse beta-cells; (2) ATP-induced transient phase of increase in  $[\text{Ca}^{2+}]_i$  in mouse beta-cells is higher but shorter than that in rat beta-cells; (3) transient phase of increase in  $[\text{Ca}^{2+}]_i$  is due mainly to  $\text{Ca}^{2+}$  release from intracellular  $\text{Ca}^{2+}$  pools whereas sustained phase of increase in  $[\text{Ca}^{2+}]_i$  is caused by  $\text{Ca}^{2+}$  influx; (4) ATP induces the increase in  $[\text{Ca}^{2+}]_i$  in beta-cells via its cell-surface P2 receptor; (5) Glucose induced increase in  $[\text{Ca}^{2+}]_i$  in mouse beta-cells sustained for longer time than that in rat beta-cells, which may explain a high sensitivity of mouse beta-cells to glucose stimulation.

## INTERNALIZATION PROPERTIES OF THE THYROTROPIN-RELEASING HORMONE (TRH) RECEPTOR TYPE 2 DIFFER FROM THAT OF TRH RECEPTOR TYPE 1

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Since the recent identification of a second receptor for TRH (TRHR2), little is known about how this receptor is regulated, in particular the mechanism for internalization. Like other members of the G-protein coupled receptor (GPCR) family the TRHR1 internalizes via a  $\beta$ -arrestin and dynamin-dependent pathway. Therefore, the aim of this study was to determine if subtype-specific differences in the internalization process represent a mechanism for selectivity between these two receptor subtypes. TRHR2, like TRHR1, internalizes via a clathrin-mediated process as internalization was inhibited by a dynamin mutant (K44A) and hypertonic conditions. Overexpression of  $\beta$ -arrestin 1 or 2 equally promoted the internalization of TRHR1 and co-expression of G-protein coupled receptor kinases (GRKs) did not further enhance TRHR1 internalization. In contrast, internalization of TRHR2 was significantly promoted by  $\beta$ -arrestin 2 and further enhanced by GRKs. These findings were supported by confocal microscopy experiments in HEK 293 cells where addition of agonist to TRHR1 expressing cells caused a redistribution of GFP-tagged  $\beta$ -arrestin 1 and 2. However, in cells expressing TRHR2, only GFP-tagged  $\beta$ -arrestin 2 showed an agonist-dependent redistribution. In addition, the trafficking of GFP-tagged  $\beta$ -arrestin 2 differed between the TRHR subtypes. Longer treatments with TRH in cells expressing TRHR1,  $\beta$ -arrestin 2 formed intracellular vesicular like structures, but remained at the plasma membrane in TRHR2 expressing cells. A quantitative assessment of the differential  $\beta$ -arrestin-dependence of TRHR2 in HEK 293 cells was carried out using bioluminescence resonance energy transfer (BRET), which measures protein-protein interactions in living cells. A similar BRET signal was obtained for the interaction between TRHR1 and  $\beta$ -arrestin 1 or, TRHR1 and  $\beta$ -arrestin 2. However, TRHR2 gave a higher BRET signal with  $\beta$ -arrestin 2, which was significantly inhibited by co-expressing wildtype  $\beta$ -arrestin 2 but not  $\beta$ -arrestin 1. Therefore, TRHR2 internalization differs from TRHR1 in that it preferentially interacts with  $\beta$ -arrestin 2 and can utilize GRKs for this process and thus can be termed a Class A GPCR by its  $\beta$ -arrestin-dependence whilst the TRHR1 is a Class B GPCR. This study reports that internalization of TRHR subtypes are differentially regulated by the  $\beta$ -arrestins and GRKs. A further understanding of TRHR2 regulation and how this differs to TRHR1 may aid in delineating the function of this novel receptor subtype and in future studies of receptor responsiveness should a specific TRHR2 agonist be developed.

## CHARACTERIZATION OF THE MOLECULAR DETERMINANTS OF THYROTROPIN RELEASING HORMONE (TRH) RECEPTOR HOMO-OLIGOMERIZATION

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A growing body of evidence suggests that formation of receptor dimers is a general phenomenon amongst G protein coupled receptors (GPCRs), involving both non-covalent and covalent interactions, with dimerization demonstrated to occur via different receptor regions. We have recently shown in living cells using bioluminescence resonance energy transfer (BRET) that the TRH receptor (TRHR) undergoes constitutive and agonist-mediated oligomerization, as an energy transfer was detected between co-expressed Renilla luciferase (Rluc; bioluminescent donor) and EYFP (fluorescent acceptor) tagged TRHRs, which can only occur when acceptor and donor molecules are less than 50Å apart. In order to further characterize the interaction between TRHRs, the role of non-covalent interactions as well as disulfide bonds was assessed. Extracellular cysteine (Cys) residues have previously been shown for many GPCRs, including the TRHR, to be required for integrity of the ligand binding site, however their role in TRHR oligomerization is unknown. To analyse the role of disulphide bonds in TRH receptor dimer/oligomer formation, the effect of DTT on constitutive and agonist-induced TRHR BRET was analysed. In addition, Cys residues in TRHR/Rluc and TRHR/EYFP were mutated (C98A, C100A or C179A) and the effect on ligand binding, signaling and constitutive and ligand-induced BRET determined. Expression of a rGnRHR/TRHR tail chimeric construct in cells co-transfected with TRHR/Rluc and TRHR/EYFP was performed to assess the role of C-tail sequences in TRHR complex formation. The chimeric construct did not affect the constitutive BRET signal obtained between the Rluc and EYFP tagged constructs, suggesting that the C-tail is not required for TRHR dimerization. The involvement of transmembrane residues in non-covalent receptor interactions, in particular the 6<sup>th</sup> transmembrane domain was also assessed using BRET. This study examines the nature of the interaction between TRHR monomeric units and maps regions involved in receptor-receptor association.