THE MOLECULAR MECHANISMS OF INSULIN ACTION IN HUMAN THECA AND ADIPOCYTE CELLS IN POLYCYSTIC OVARIAN SYNDROME

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DECLARATION

I declare that the thesis is the result of my own work based on research that was undertaken at the Academic division of Obstetrics and Gynaecology, Derby Medical, University of Nottingham during the period of October 2008 to October 2011.

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30th August 2012

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PUBLICATIONS

Abstracts

Cadagan, D. Towlson, C. Khan, R. Amer, S. 2010 **The influence of Luteinising Hormone on Adipocyte Proliferation in Women with and without Polycystic Ovarian Syndrome.** *The Royal College of Obstetrics and Gynaecology, Blair Bell Research Symposium*

Cadagan, D. Towlson, C. Khan, R. Amer, S. 2010 **Progesterone Secretion in Adipocytes and Variations in Polycystic Ovarian Syndrome.** *1st International Symposium on Advances in Human Metabolic Research*

Cadagan, D. Warren, A. Khan, R. Amer, S 2010 **An Alternate Endocrine Function in Adipocytes with possible associations to Polycystic Ovarian Syndrome.** *9th Congress of the European Society of Gynecology*

Cadagan, D. Towlson, C. Khan, R. Amer, S. 2011 **Variations in Proliferation of Adipocytes within Normal and Polycystic Ovarian Syndrome under the effect of Insulin.** *BPS Winter Meeting 2011.*

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ABSTRACT

PCOS is one of the leading causes of infertility worldwide affecting 1 in 10 women of a reproductive age. One of the fundamental abnormalities in women with PCOS can be seen within hormonal irregularities, which may include hyperandrogenemia hyperinsulinemia and hyper secretion of luteinising hormone (LH); and it is hypothesised that a defect in steroid secreting ovarian theca cells is involved due to their contribution in non-PCOS hormonal synthesis.

Hyperinsulinemia has been associated with hyper-androgenemia through *in vitro* studies of cultured PCOS theca, where it has been suggested that insulin increases progesterone and androstenedione secretion when compared to normal theca cells. Furthermore the augmented effects of LH and insulin have been seen to increase ovarian androgen synthesis in non-PCOS theca cultures whilst also increasing the expression of steroidogenic enzymes specific to the PI3-K pathway.

Many theories exist toward the etiology of hyper androgenemia within PCOS. Very few approaches however, consider dysfunction in multiple tissue types that may contribute to hormonal imbalances. It is well established that an association between obesity and PCOS exists and it is often the first therapeutic target for re-establishing reproductive function in obese PCOS patients. Furthermore PCOS patients tend to show distinct gynoid body fat distribution, which is reported to aggravate PCOS symptoms. It was therefore valid to examine the involvement in adipocyte function and its contribution to androgen levels within PCOS. This is further supported through the link between metabolic disorders such as insulin resistance and hyperinsulinemia, and their associations to obesity.

Our study employed isolated preadipocyte and thecal cultures with close regulation of the influential factors LH and insulin. In doing so, we analysed androgen synthesis through activation and expression of steroidogenic enzymes CYP17 within both normal and polycystic ovaries. This allowed us to examine whether protein/hormonal concentrations vary across non-PCOS and PCOS cultures. This also allowed us to examine the possibility of a novel pathway leading to localised adipocyte synthesis as

well as pinpointing whether dysfunction existed within the insulin-signalling pathway of thecal androgen steroidogenesis.

The work in this thesis shows that adipocytes derived from non-PCOS and PCOS women, maintained *in vitro* differ on the basis of their morphology, rates of differentiation and proliferation. Furthermore, they reacted differently under conditions designed to mimic PCOS *in vitro* (increased insulin and LH), with reduced non-PCOS proliferation, and increased non-PCOS androgen secretion on insulin treatment. We also found increased steroidogenic CYP17 expression in PCOS cultures under insulin stimulation. However PCOS adipocytes androstenedione secretion remained unaffected by insulin stimulation and secreted constant levels of androstenedione similar to that seen by insulin stimulated non-PCOS adipocytes.

Our examination of non-PCOS and PCOS primary thecal cultures showed CYP17 expression is increased in PCOS theca under basal conditions and that increases in insulin and LH leads to increases in *in vitro* theca proliferation. These conditions were also seen to lead to significant increases in androstenedione secretion over non-PCOS thecal cultures, and the results suggest it to be acting through the PI3-K pathway. These results therefore point to a specific area of dysfunction that should be further targeted for examination. Furthermore, they suggest that an adipocyte dysfunction exists within PCOS patients that may significantly contribute to hyperandrogenemia through localized synthesis of androgens.

ABBREVIATIONS

- 2-βME-2-β mercaptoethanol
- 3T3-L1-Swiss murine cell line
- 3β-HSD-3β-hydroxysteroid
- 11β-HSD-11β-hydroxysteroid
- 17β-HSD-17β-hydroxysteroid
- 17,20-lysase-see CYP17
- AA-Arachidonic acid
- ACE- Angiotensin converting enzyme
- ACh-Acetylcholine
- Akt-see PKB
- ANOVA-Analysis of variance
- AP-Alkaline Phosphatase
- AP-2
- Apo E-Apoprotein E
- AKR1C2/3-Aldo Keto reductase 1/2
- ATP- Adenosine trisphosphate
- AR-Androgen receptor
- ASRM- American Society for Reproductive Medicine
- BCA-Bicinchoninic Acid
- BMI-Body mass index
- BSA-Bovine serum albumin
- CO2- Carbon dioxide
- CHO-Chinese hamster ovary cells
- COX- Cyclooxygenase
- CRH- Corticotrophin releasing hormone
- Cu2+- Copper ion
- CuCl2-Copper chloride
- CURC-Curcumin
- Cyclic AMP-cyclic adenosine 3'5' monophosphate
- Cyclic GMP-cyclic guanosine 3'5'monophosphate
- CYP17-17α-hydroxylase
- CVD-Cardiovascular disease
- DAB -3,3' Diaminobenzidine
- DHEA- Dehydroepiandrosterone
- DHEAS- Dehydroepiandrosterone sulphate
- DHT- Dihydrotestosterone
- DMEM-Dulbecco's modified eagle medium
- DMSO-Dimethyl sulfoxide
- DNP-2,4-Dinitrophenol
- ECM-Extra cellular matrix
- EDTA-Ethylenediaminetetraacetic acid
- ER-endoplasmic reticulum
- ERα-Eostrogen receptor alpha

- ERK-see MAPK
- ESHRE- European Society of Human Reproduction and Embryology
- EtOH-Ethanol
- FAI-Free androgen index
- FBS-Fetal bovine serum
- FITC-Fluorescein Isohiocynate
- FSH-Follicular stimulating hormone
- GnRH-Gonadotropin releasing hormone
- GLUT4-Glucose transporter (type 4)
- GTP- guanosine triphosphate
- GTT-Glucose tolerance test
- HBSS -Hank's Balanced Salt Solution
- HCl-Hydrochloric acid
- HCG-Human chorionic gonadotropin
- HDL-High density lipoprotein
- HPO-Hypothalmic pituitary axis
- IBTX-Iberiotoxin
- IF-Immunofluorescence
- IGF-1-Insulin like growth factor-1
- IGBP-1-Insulin like growth factor binding protein-1
- IHC-Immunohistochemistry
- IKCa-Calcium activated intermediate conductance potassium channel
- IL-6-Interleukin 6
- IQR-Interquartile range
- IR-Insulin receptor
- K+- Potassium ion
- kDa- Kilo Dalton
- LDL-Low density lipoprotein
- LH-Luteinising hormone
- L-METH-L-Methionine
- LPL-Lipoprotein lipase
- MAPK-Mitogen activated protein kinase
- MB-Methylene blue
- MEM-Minimum essential media
- MMP-Matrix metalloproteinases
- mRNA- Messenger ribonucleic acid
- M-WU test-Mann Whitney U test
- NADPH- Nicotinamide adenine dinucleotide phosphate
- Na+-Sodium ion
- NaCl-Sodium chloride
- NaCN- Sodium cyanide
- NAD-Nicotinamide
- NIH-National institute of health
- NICHD-National Institute of Child Health and Human Development
- NORM-Normal
- ns- not significant
- P450arom-Aromatase

- P450C17-see CYP17
- P450scc- Enzyme P450 side chain cleavage
- PBS-Phosphate Buffered Saline
- PCOS-Polycystic ovarian syndrome
- pCO2-Pressure of CO2
- PDK- phosphotidylinositide-dependent protein kinase
- PFA-Paraformaldehyde
- PG-Prostaglandin
- PGI2-Prostacyclin
- PI3-K-Phosphatidylinositol 3-kinases
- PKA- Protein
- PKB- protein kinase B
- pO2-Pressure of oxygen
- PSS-Physiological salt solution
- RAS-Renin angiotensin system
- RDH5-Retinol dehydrogenase
- RIL-Riluzole
- RM-ANOVA-Repeat measures ANOVA
- ROI-Region Of interest
- ROS-Reactive oxygen species
- RT-Room temperature
- RT-PCR-Reverse transcription polymerase chain reaction
- SC-Subcutaneous fat
- SCP-2- Sterol carrier proteins
- SD-Standard deviation
- SDS-PAGE- Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
- SEM-Standard error of the
- SHBG-Serum hormone binding globulin
- StAR- Steroidogenic acute regulatory protein
- T2D-Type 2 diabetes
- TBS-Tris Buffered Saline
- TBS-T-Tris Buffered Saline + Tween
- TNFα-Tumour necrosis factor alpha
- WHR-Waist to hip ratio
- Zn2+- Zinc ion
- ZnCl2-Zinc chloride

1.0 INTRODUCTION

Polycystic ovarian syndrome (PCOS) affects approximately 10% of all women of a reproductive age and is one of the most commonly occurring endocrinopathy. PCOS is recognised as a reproductive disorder and manifests through infertility, chronic anovulation, and hyperandrogenism (*see 1.6*). Pathogenesis is yet to be determined.

1.1 Structure and function of the ovaries

Understanding ovarian anatomy, morphology and typical biochemical function, is essential to understanding polycystic ovarian syndrome as an endocrinological disorder as well as its relationship to infertility.

The ovaries are the female organs essential to fertility through their production of ova and synthesis of the hormones progesterone and oestrogen. These hormones are responsible for sexual development and regulation of the menstrual cycle through their ability in such actions as stimulation of endometrial and uterine growth [1].

Mesentery membranes from the broad ligament of the uterus suspend the ovaries (**Figure 1**). They also support and house the ovarian artery, ovarian vein, lymphatic vessel and nerve fibres. Each ovary rests within a depression on the ovarian fossa toward the lesser pelvis and are around 2cm/4cm across decreasing in size with the age of the individual [2, 3].

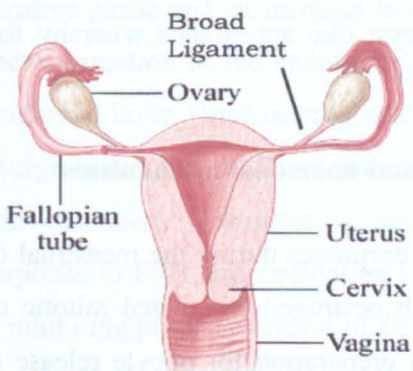

Figure 1. Female Reproductive System showing the major organs including the cervix, uterus and ovaries. (Diagram adapted from http://3.bp.blogspot.com)

The outer structure of the ovary consists of columnar cuboidal cells forming the superficial epithelium, which alters at the hilum, the point of attachment for the mesentery. The internal ovarian structure combines the outer portion, cortex and inner medulla (**Figure 2**).

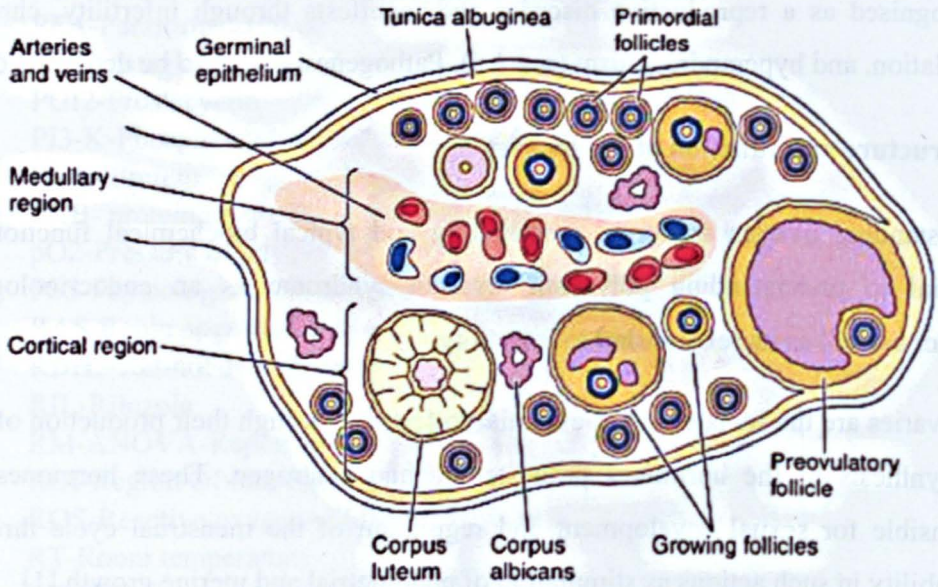

Figure 2. Anatomy of the ovary showing stages of follicular development. Picture adapted from //www.mc.vanderbit.edu

The outer portion consists of thin connective tissue, tunica albuginea, allowing a spongy protective membrane. The cortex is made up of stromal cells creating a fibre network, follicles and corpora lutea. The cortex supports germinal, endocrine and vascular elements such as the follicles or the corpus luteum. Typically within PCOS this surface area shows a cyst like appearance whereby follicles are halted during development (*see* **Figure 14**).

1.1.2 The menstrual cycle and hormonal mechanisms

The orchestrated release of hormones during the menstrual cycle allows the build up of the endometrium through oestrogen stimulated mitotic division of the glandular epithileum in the stroma in preparation for oocyte release and possible fertilisation during conception. Failing fertilisation the lining is shed (menses) due to a reduction in oestrogen and progesterone which leads to constriction of spiral arteries and subsequent tissue necrosis [4]. The menstrual phase lasts, on average from 2 to 7 days [5]. The key endocrine features of the menstrual cycle are illustrated in **Figure 3**.

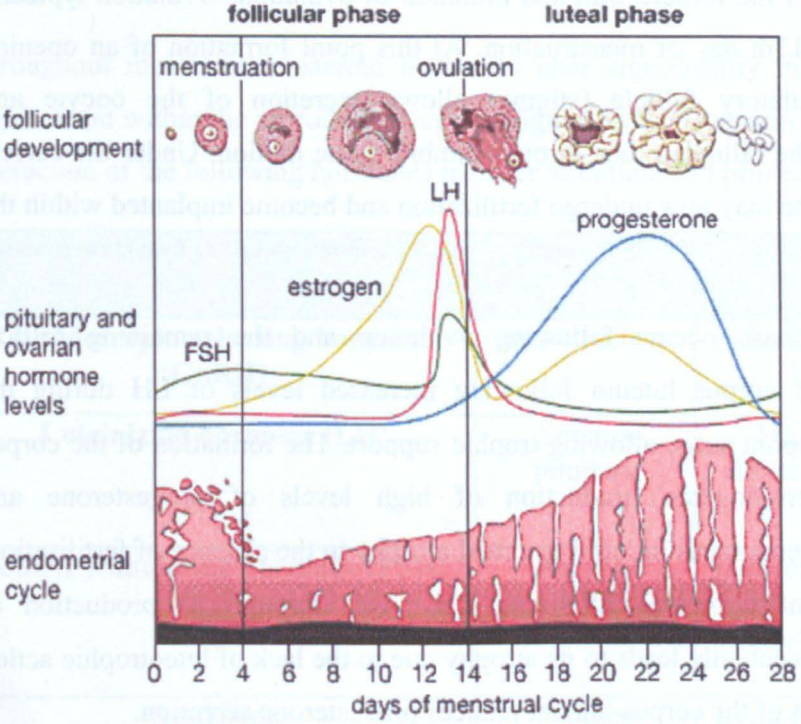

Figure 3. Concentrations of pituitary and ovarian hormones during the menstrual cycle. The chart shows the varying levels of key hormones required within the menstrual cycle and their relationship to the stages of follicular development. (Adapted from the http://www.britannica.com/EBchecked/topic/498625/human-reproductive-system).

The menstrual phase is followed by the follicular phase, whereby the rise in follicular stimulating hormone (FSH) allows folliculogenesis (*see 1.2*). The follicular phase is also known as the proliferative phase and an increase in oestrogen secretion through folliculogenesis creates new formation of the endometrial lining generated from the stratum basale, or base epidermal layer. Once oestrogen levels reach their threshold, a midcycle gonadotrophin surge occurs which allows maturation of the ovum within the pre-ovulatory follicle and ovulation. Oestrogen is secreted increasingly via the developing follicles in response to FSH and circulating levels increase. At threshold negative feedback occurs inhibiting pituitary release of FSH. At this point the follicles begin atresia whilst the most developed follicle develops receptors for luteinising hormone (LH) and FSH allowing sufficient support for maturation. The mature follicle continues to increase estradiol secretion allowing positive feedback. This results in hypothalamic gonadotropin releasing hormone (GnRH) secretion and subsequent high-level pituitary secretion of LH and FSH (midcycle surge). LH release

allows weakening of the follicle wall and initiation of ovulation. Ovulation typically occurs around the 13th day of menstruation. At this point formation of an opening within the pre-ovulatory follicle (stigma) allows excretion of the oocyte and progression down the fallopian tube through fimbra tissue motion. Under the correct conditions the oocyte may now undergo fertilization and become implanted within the uterus [5, 6]

The final luteal phase occurs following ovulation and the remaining follicle transforms into the corpus luteum following increased levels of LH during the midcycle gonadotrophin surge allowing trophic support. The formation of the corpus luteum allows steroidogenic production of high levels of progesterone and maintenance of the endometrium until eventual atrophy in the absence of fertilisation. The corpus luteum suppresses FSH and LH levels through its production of progesterone, this eventually leads to its atrophy due to the lack of luteotrophic action via LH. Degradation of the corpus luteum reduces progesterone secretion.

The luteal phase, also known as the secretary phase, incorporates decreasing levels of progesterone and triggers menstruation along with the shedding of the endometrial lining. This is a result of insufficient levels of progesterone supporting maintenance. With the absence of progesterone, LH and FSH levels are able to increase and in doing so trigger degradation of the corpus luteum. This in turn leads to its transformation through breakdown, to the corpus albicans. This is seen as scar tissue and is eventually re-absorbed by the ovary.

1.1.3 Key hormones within the menstrual cycle

Throughout menstruation steroid hormones alter significantly in response to their requirement within the reproductive cycle (**Figure 3**). The menstrual cycle utilises the interaction of the following hormones in order to initiate and proceed:

Hormones related to the menstrual cycle	Produced	Menstrual function
Gonadotropin releasing hormone (GnRH)	Hypothalamus	Stimulation of FSH and LH secretion
Luteinizing hormone (LH)	Anterior pituitary	Maintaining luteal function and triggering ovulation
Follicle stimulating hormone (FSH)	Anterior pituitary	Induces proliferation and development of folliculogenesis/gametogenesis
Oestrogen	Ovaries	Reproduction and sexual development acting as a growth hormone for tissue (endometrium) and is vital within
Estriol	Placenta	Reproduction and sexual development
Progesterone	Ovaries	Steroid hormone involved in the menstrual cycle and pregnancy
Androgen	Ovaries and adrenals	Sexual development, regulation of liver and kidneys

Table 1. Hormones relating to the menstrual cycle.

Gonadotropin releasing hormone (GnRH). Synthesized and released in low frequency pulses from the hypothalamus and is responsible for the stimulation of FSH and LH secretion. It has been acknowledged that hyperinsulinemia found in PCOS encourages increased pulsatile secretion and results in increased LH levels (*see* **Table 1** *and 1.1.2.*).

Luteinising hormone (LH). Produced in the anterior pituitary and responsible for maintaining luteal function and triggering ovulation. It also plays a key in the

hypothalamic pituitary ovarian axis (HPO axis) in its actions in ovulation and luteolysis (*see* **Table 1** *and 1.4*) [7].

Follicle stimulating hormone (FSH) is stimulated by the anterior pituitary gland and is responsible for regulation of follicle growth and development. FSH also induces proliferation and development of folliculogenesis/gametogenesis along with LH/FSH receptor expression within theca/granulosa during menstruation and the following follicular phase (*see* **Table 1** *and 1.4*) [8] .

Oestrogen. The primary female sex hormone exist in three forms estrone, estradiol and estriol. Estrone is secreted via the ovaries and stored in fatty tissue. Estrone is typically stored for later conversion to its more active form estradiol [9, 10]. Estradiol is the predominant sex hormone produced within the ovaries and responsible for many functions within the human body including; liver lipoprotein synthesis, brain antioxidant formation and bone maintenance. It is also vital within female reproduction and sexual development acting as a growth hormone for tissue (endometrium) and is vital within folliculogenesis for development (*see 1.6*) [9]. Estriol is only produced during pregnancy and can be used as either a weak or strong oestrogen.

Progesterone. Derived from cholesterol through the conversion of pregnenolone. It is a steroid hormone involved in the menstrual cycle and pregnancy and is vital for initiation and maintenance of the endometrium along with initiation of luteal phase of menstruation (*see* **Table 1** *and 1.4*).

Androgens. These are vital to female metabolism produced predominately via the ovaries and adrenals and allow hormonal cascades such as puberty to be initiated in both males and females. They play key roles in regulation of organs including the liver and kidneys [11], and are important in muscle and bone development [12, 13]. Female androgens consist of dehydroepiandrosterone sulphate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione, dihydrotestosterone (DHT) and testosterone. DHEAS, DHEA and androstenedione are considered pro androgens, precursors to testosterone with weak androgenic effect (**Figure 4**).

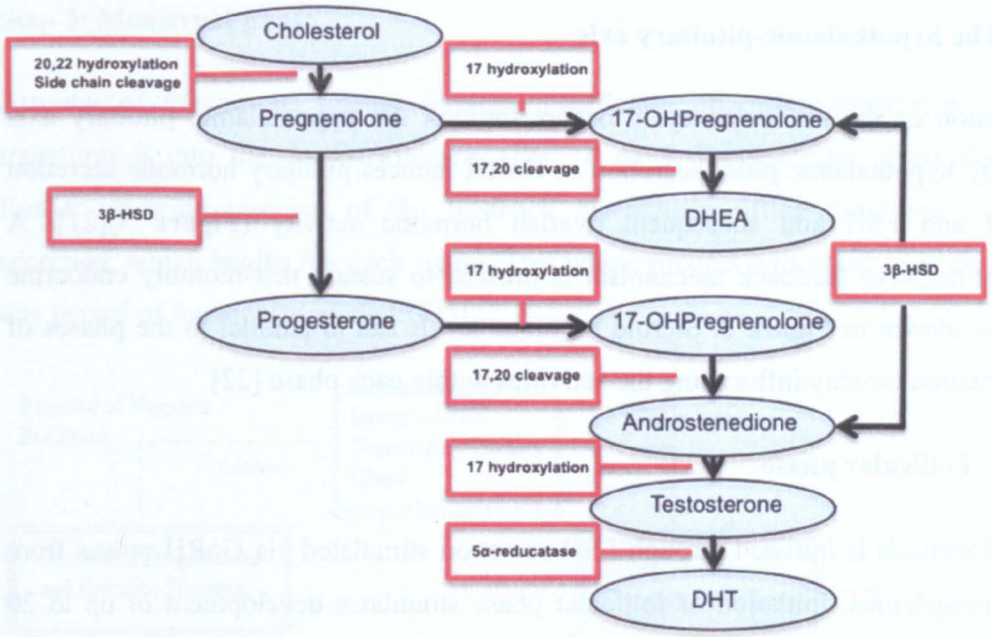

Figure 4. Female Androgen Synthesis within the Ovaries. (Adapted from Rosenfield, 1999a).

Increased plasma testosterone and androstenedione has been seen during the mid cycle phase of the menstrual cycle. This suggests increased levels of androgen synthesis exist at this stage supporting its links to sexual health and maintenance (*see 1.4*) [14-16]. The functions of androgens are physiologically diverse, and can have effects on regulation of metabolic activity including proliferation and biosynthesis. This has been seen within many tissue types including adrenal, muscle and adipose tissues [17-19]. Androgens are also regarded as a steroidogenic precursor necessary in the biosynthesis of testosterone and oestrogen [20].

1.1.4 The hypothalamic-pituitary axis

Regulation of the menstrual cycle occurs through the hypothalamic pituitary axis whereby hypothalamic pulse secretion of GnRH induces pituitary hormone secretion of LH and FSH and subsequent ovarian hormone activity (**Figure 3**)[21]. A positive/negative feedback mechanism is utilized to sustain this monthly endocrine cycle as shown in **Figure 5**. Steroid hormone levels run in parallel to the phases of menstruation directly influencing the activities within each phase [22].

Step 1: Follicular phase

folliculogenesis is initiated through FSH secretion stimulated via GnRH pulses from the hypothalamus. Initiation of follicular phase stimulates development of up to 20 follicles per cycle allowing increased follicular secretion of oestrogen. Rising estradiol levels exert negative feedback on the hypothalamus decreasing GnRH secretion. Negative feedback is also present at the pituitary, decreasing its sensitivity to GnRH, resulting in reduced FSH secretion, which inhibits further follicle development (*see 1.4*). All but one follicle undergoes atresia due to the reduced levels of FSH/LH, however, the dominant follicle up-regulates FSH/LH receptors facilitating further maturation [5, 21, 23].

Estradiol eventually reaches a threshold causing positive feedback on the hypothalamus, at which point an LH surge occurs (**Figure 3**), which acts on the dominant follicle. The LH surge allows maturation of the egg and rupture of the follicle wall through luteinisation. This stimulates ovulation and the release of the oocyte into the fallopian tube. [24]

Step 2: Luteal phase

The remaining ruptured follicle transforms under the influence of FSH/LH, into the corpus luteum. Transformation allows increased progesterone and oestrogen secretion leading to suppression of FSH and LH (negative feedback). Failing fertilisation, progesterone will reach threshold. The decrease in concentration removes FSH/LH inhibition and these conditions will result in menstruation (**Figure 3, Figure 5**).

Step 3: Menstrual phase

Atrophy of the corpus luteum through insufficient progesterone/estradiol levels; transform it into the corpus albicans after 14 days. Progesterone levels decrease further allowing removal of the feedback mechanism lifting inhibition of FSH secretion, which begins the cycle again. This whole process will occur over the 28-32 day period of the menstrual cycle [25].

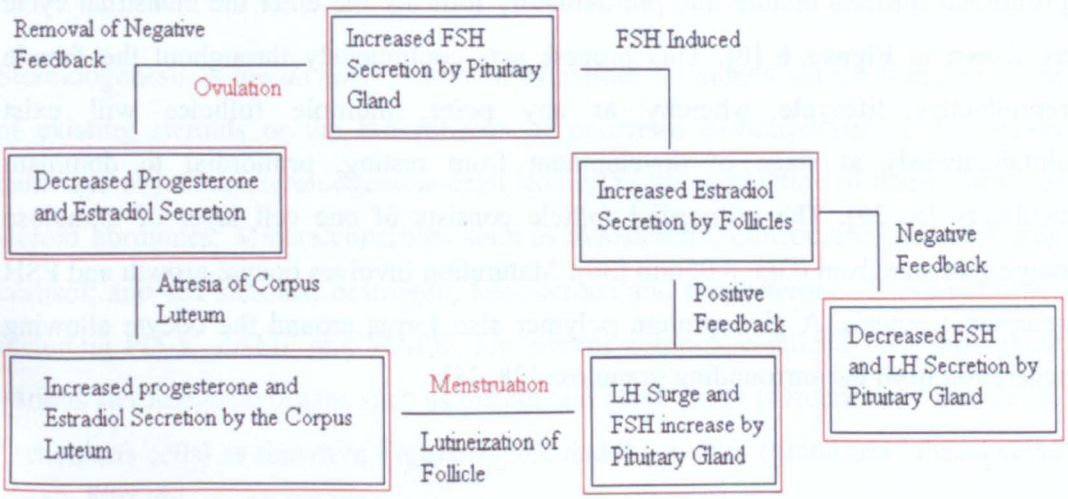

Figure 5. Positive and Negative feedback mechanisms of menstrual hormones. The flow chart shows positive and negative feedback mechanisms and their effect on hormonal secretions during the menstrual cycle.

1.2 Folliculogenesis

The ovaries are responsible for dual functions of steroid hormone synthesis and gamete production (*see 1.3.1*)[26]. The follicles and corpora lutea are the main ovarian structures vital to reproduction (*see* 1.4). From birth, the ovaries contain a set number of immature primordial follicles (around 7 million) each containing an immature oocyte [24, 27, 28]. Folliculogenesis is the process by which small primordial follicles mature into pre-ovulatory follicles and enter the menstrual cycle as shown in **Figure 6** [6]. This process acts continuously throughout the female reproductive lifecycle whereby at any point, multiple follicles will exist simultaneously at stage of development from resting, primordial to dominant ovulatory [6, 29]. The primordial follicle consists of one cell layer of granulosa ranging in size from 0.03–0.05mm [30]. Maturation involves oocyte growth and FSH receptor synthesis. A glycoprotein polymer also forms around the oocyte allowing separation from the surrounding granulosa [28, 31].

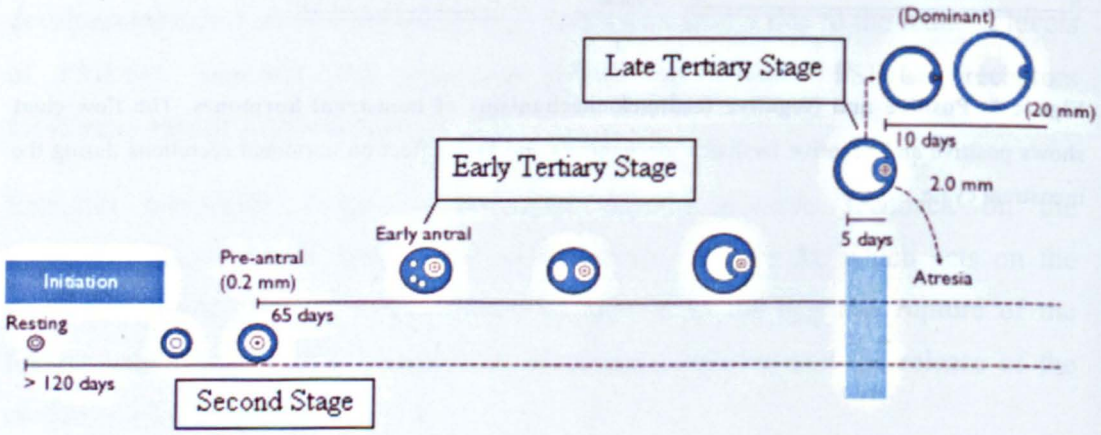

Figure 6. 375-day cycle of folliculogenesis showing the follicle progression through development from resting to dominant. (Diagram adapted from http://www.ncbi.nlm.nih.gov /).

During the second stage of folliculogenesis, granulosa proliferation increases the follicle to 0.01mm in diameter. Continued development allows the emergence of a fibrocellular envelope of thecal cells surrounding multiple layers of granulosa and doubling the follicles size to 0.2mm (**Figure 6**). At this point, formation of the antrum occurs cushioning the oocyte in a fluid cavity [28, 29]. This is known as the tertiary phase, which is considered in two parts; early (graafian) and late. The secretion of

LH from the pituitary (*see 1.1.3*) begins allowing the stimulation of androgen formation and eventually leading to oestrogen production through aromatization within the granulosa cells (*see 1.3.2*). Late tertiary phase is only reached by the primary follicle which progresses to pre-ovulatory through the secretion of oestradiol and FSH signalling. At this stage the follicle can reach 20mm in size before entering ovulation.

1.3 Steroidogenesis

Steroidogenesis is the *de novo* formation of steroid hormones via the transformation of existing steroids or the biosynthesis of precursor carbohydrates [25]. Various pathways of human steroidogenesis exist leading to the production of three classes of steroid hormones: Mineralcorticoids such as aldosterone; Glucocorticoids, including cortisol; and sex steroids, oestrogen, testosterone and progesterone. These pathways including PI3-K, cAMP and MAPK, are present within specialized cells throughout various steroidogenic organs such as the adrenal gland (zona glomerulosa / fasciculate / reticularis cells) as shown in **Figure 7** [32], and the ovaries (granulosa / thecal cells) as shown in **Figure 10** [20, 33].

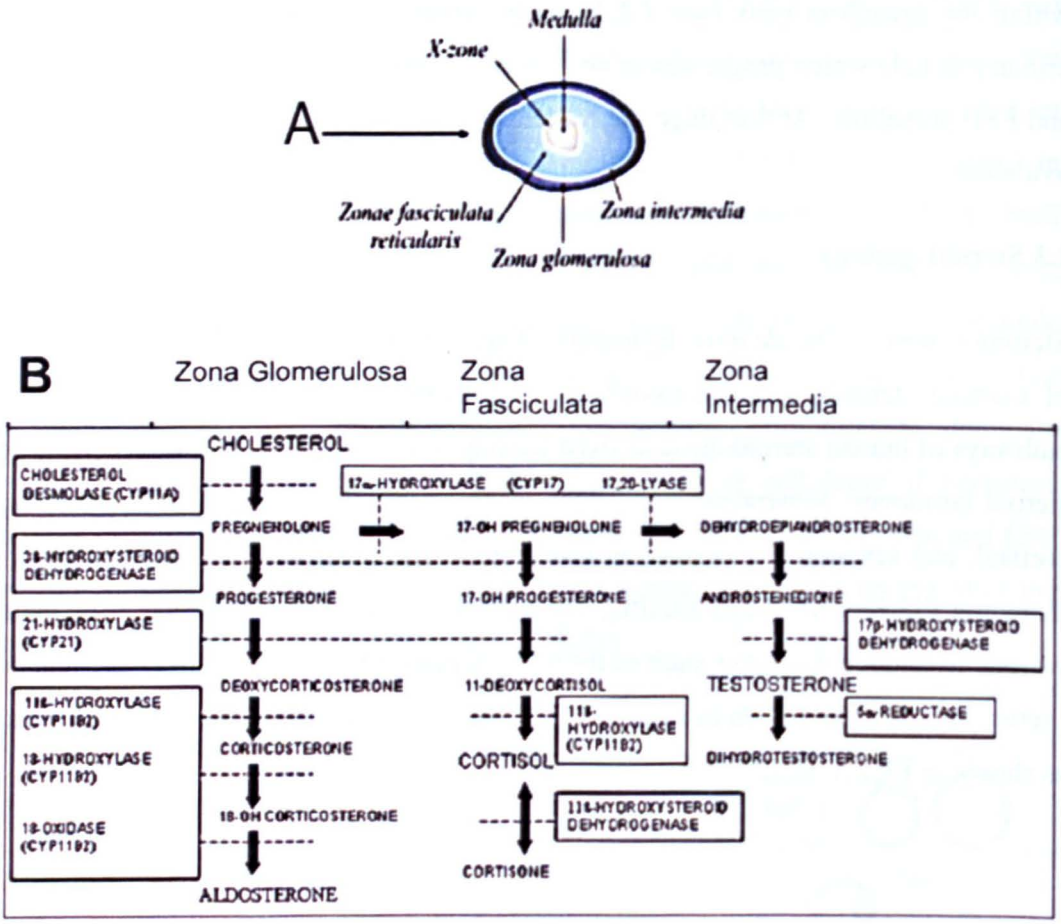

Figure 7. (A) Compartmentalisation of adrenal cell (B) Adrenal cell steroidogeneic pathway. The adrenal cell is made up of the Zona glomerulus, fasciculata and intermedia (A) where steroidogenesis occurs through compartmentalisation. (Diagram adapted from www.endotext.org/adrenal/adrenal26/adrenal26.)

Ovarian function is dependent on the combined action of LH on thecal cells and FSH on the granulosa cells activating biosynthetic pathways (**Figure 5**) [1]. In response the 'Two cell model of ovarian steroidogenesis' dictates transportation of thecal steroidogenic products to the granulosa where, through the influence of FSH on granulosa, activation of many gene, promoting proliferation and differentiation occurs including MAP2D, inhibin-α and VEGF [34]. Furthermore FSH stimulates up-regulation of LH receptor and aromatase expression and therefore encourages, oestrogen formation (**Figure 10**) [9]. Eventually the required androgen synthesis decreases due to ovulation and inhibition of FSH secretion occurs through increased

progesterone levels (*see 1.4*). This prevents maturation and initiates atresia of the undeveloped follicles [27, 35].

1.3.1 Regulation and transportation of steroidogenic precursors

Cholesterol conversion occurs within the mitochondria as the first rate limiting stage of steroidogenesis. Regulation is the result of cholesterol transportation via steroidogenic acute regulatory protein (StAR) to the internal mitochondrial membrane (**Figure 8**.)[36].

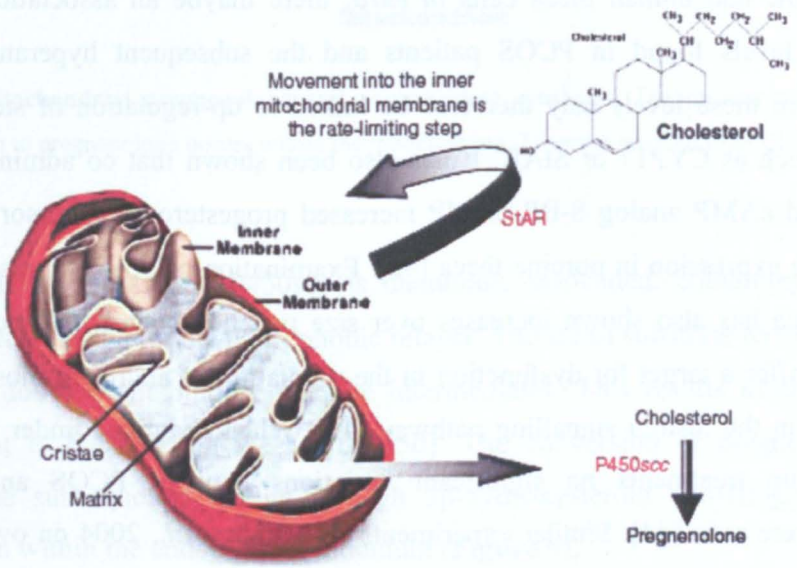

Figure 8. Cholesterol transportation to mitochondria within steroidogenesis. The importance of Star as a transport protein for movement of cholesterol across the mitochondrial membrane can be seen. (Diagram adapted from Arukwe, 2008).

The two stage process begins with sterol transfer to the mitochondria through sterol carrier proteins (SCP-2)[37]. This stage is seen as non-essential, with compensatory methods of transportation existing in the absence of SCP-2. Typically within gonadal and adrenal steroidogeneisis, transport occurs through StAR activity [38]. Increased StAR concentrations are found in the absence of SCP-2 supporting its importance as a primary transportation mechanism [39].

Stage two sees cholesterol translocation from the cholesterol rich exterior mitochondrial membrane, to the cholesterol poor interior. This process again utilises StAR which is required to translocate the largely hydrophobic cholesterol across the

aqueous phase between membranes [40]. The exact mechanism of cholesterol translocation is poorly understood. Structural alterations within the mitochondrial membrane are believed to allow cholesterol passage to its interior. However a loose association of StAR to its exterior exists during transportation [39, 40]. This may suggest StARs role in a larger molecular pathway through the inefficiency of this mechanism.

Regulation of thecal steroidogenesis is primarily through LH action acting through cAMP dependent protein kinase A (PKA) [41]. With insulin and LH now believed to act both independently and synergistically in androgen synthesis when examined in both porcine and human theca cells *in vitro*, there maybe an association with the increased levels found in PCOS patients and the subsequent hyperandrogenmia. Furthermore these levels may therefore be linked to up-regulation of steroidogenic proteins such as CYP17 or StAR. It has also been shown that co administration of insulin and cAMP analog 8-BR-cAMP increased progesterone precursor as well as StAR gene expression in porcine theca [42]. Examination of StAR expression within PCOS theca has also shown increases over size matched control follicles and may therefore offer a target for dysfunction in the regulation of androgen biosynthesis at this stage in the insulin signalling pathway [43]. When examined under insulin and LH/forskolin treatments no significant variations between PCOS and matched controls were seen [44]. Similar experiments by Munir *et al.*, 2004 on ovarian theca of non-PCOS patients have shown increases in CYP17 expression and activity under insulin, LH and forskolin treatments [45] making its position in androgen biosynthetic pathway our focal point for a possible dysfunction in PCOS

1.3.2 Steroidogenesis: substrate conversions

The conversion of cholesterol to pregnenolone occurs in three chemical phases: 20 α-hydroxylation, 22-hydroxylation and scission of the cholesterol side chain. P450scc is the single enzyme required for all three reactions [46, 47]. The first two chemical reactions hydroxylate the carbon atom at its structural points C-20 and C-22 before finally cleaving these bonds [48].

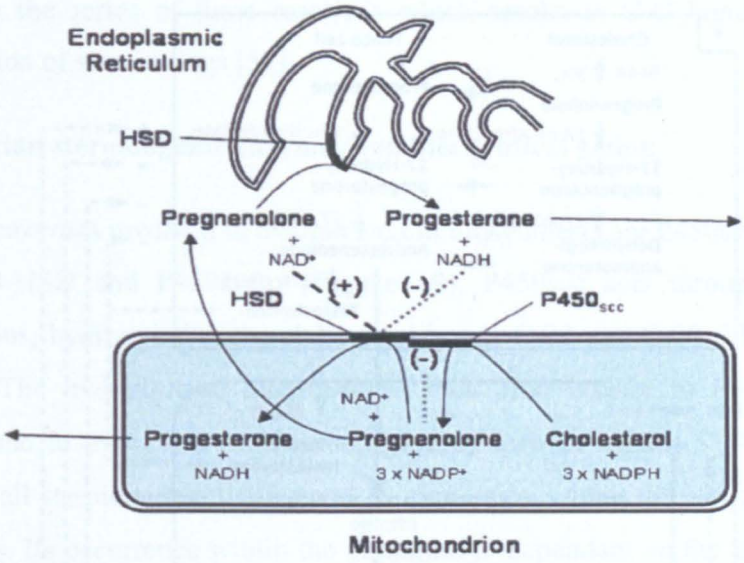

Figure 9. Mitochondrial pregnenolone and progesterone synthesis. The process of cholesterol transformation to pregnenolone occurs within the mitochondria (Diagram adapted from Chapman et al., 2005)[49].

As with all P450 enzymes, P450scc is membrane associated, containing a single active site in contact with the hydrophobic bilayer. The sterol substrate remains bound to the site due to tight binding reactive intermediates. This results in cholesterols commitment to the reaction (*see 1.10.2*)[50]. The conversion of pregnenolone to progesterone subsequently occurs through 3β-hydroxysteroid dehydrogenase (3β-HSD) action within the endoplasmic reticulum (**Figure 9**).

Research into the expression of both P450scc and 3β-HSD in *in vitro* PCOS theca against matched controls has shown no variation in mRNA levels under basal conditions [51]. However Nelson *et al.,* 2001 demonstrated that under forskolin stimulation 3β-HSD mRNA expression is increased in non-PCOS theca cells and still further in *in vitro* cultures of theca obtained from women with PCOS, suggesting a possible increase in PCOS sensitivity to LH [52]. Further evidence however by Comim *et al.,* 2011 recently examining both 3β-HSD and CYP17 protein expression through quantitative immunohistochemistry concluded no significant variation in 3β-HSD expression between non-PCOS and PCOS theca cells [53] . Their results do however provide consistent evidence pointing to abnormal levels of CYP17 expression within PCOS theca cells, which suggests its more primary involvement in thecal dysfunction.

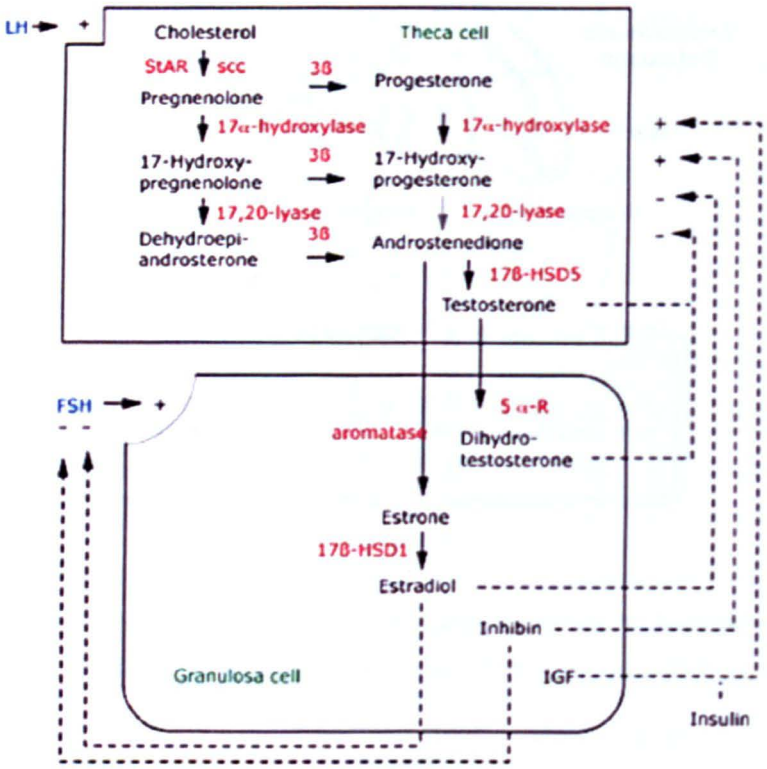

Figure 10. Steroid biosynthetic pathways, two-cell model of ovarian steroidogenesis. (Diagram adapted from Rosenfield, 1999a).

Steroid biosynthesis relies on the availability of precursors and enzymatic concentrations along with hormonal and cellular regulation [54]. Pregnenolone is the precursor for the synthesis of all ovarian steroids and biosynthesis is dependent on its conversion by steroidogenic enzymes such as CYP17 (17α- hydroxylase and 17,20-lyase) and steroid oxido-reductase. Some of these enzymes are not limited to single processes within a pathway as shown within **Figure 10**. CYP17 is active within both ovarian steroidogenesis and adrenal steroidogenesis [9, 55, 56] and has been associated to hyper secretion within ovarian steroidogeneisis. This has led to its current focus in PCOS research, and to date no variations in expression or activity have been reported for adrenal biosynthesis.

All tissues require P450 enzymes, allowing the metabolism of small hydrophobic molecules involved in metabolic and biosynthetic reactions [57]. Steroidogenic P450s resemble these with similarities such as membrane association and active heme sites for substrate O_2 and CO_2 binding. O_2 and CO_2 compete for these binding sites and can subsequently inhibit (CO_2) or activate (O_2) [47, 57]. Steroidogenic P450s do, however, differ in their mono oxygenation catalysation of specific parts of molecules (steroids),

and within the series of these reactions which results in C-C bond cleavage and aromatization of steroid rings [57].

1.3.3 Ovarian steroidogenic enzymes and biochemical action

The main enzymes involved in ovarian steroid biosynthesis are P450scc, P450c17 3β-HSD, 17B-HSD and P450arom (**Figure 10**). P450scc acts through three mono oxygenations, hydroxylating the cholesterol bonds C-22 and C-20 followed by their cleavage. The hydroxylated intermediates then bind readily to P450scc, causing pregnenolone to dissociate from the enzyme as seen in **Figure 9** [40]. P450scc is present in all steroidogenic tissues and its expression within the ovaries is typical to thecal cells. Its occurrence within the granulosa is dependent on the phase of follicle development and P450scc can increase a hundred fold within the corpus luteum [49]. Increased levels of both P450scc and 3B-HSD mRNA have also been seen during this phase suggesting enhanced transcription and their importance in progesterone synthesis. This increase is believed to relate to gonadotropin activation; with mitochondrial hydroxysteroid dehydrogenase (HSD) activity highest at the peak of LH secretion during ovulation [40, 49, 58]. With hypersecretion of LH typically associated with PCOS the importance of both P450scc and 3B-HSD in the regulation of androgen synthesis can be seen, however as yet no variation between PCOS and matched controls in either adrenal or ovarian androgen pathways has been determined (*see 1.3.2*).

CYP17 (P450c17 / 17α-hydroxylase/17,20 lyase/17,20 desmolase) is a cytochrome located within the cytosol and associated with enzymatic action on pregnenolone and progesterone, specifically within the PI3-K pathway (*see 1.4.2*)(**Figure 10**). It has been shown that LH stimulates CYP17A1 mRNA expression *in vitro* porcine theca with similar results seen using forskolin in cultured human theca cells. These findings therefore suggest that LH may be associated with increased androgen synthesis and work by McAllister *et al.*, 1989 has shown this to be true in non-PCOS human cultured theca [59, 60]. CYP17 is the steroidogenic enzyme responsible for two key reactions; 17α-hydroxylation of C-21, typically related to ovarian steroid biosynthesis, and cleavage of C17-C20 bond of C-21 steroids (**Figure 10**). The concentrations of CYP17 correlate to the stage/size of pre-ovulatory follicles with increased CYP17 found with increased follicle size [45, 57]. CYP17 has substrate species specifity. For

example rat CYP17 has the ability to convert 17-OH-pregnenolone and 17-OH-progesterone into DHEA and androstenedione allowing determinations of androgen biosynthesis via pregnenolone or progesterone whereas human CYP17 can only cleave the 17-OH-pregnenolone bond [46]. This limits the parameters of the human androgenic pathway but at the same time emphasises the possible versatility shown in other species.

Ovarian 3β-HSD exists within the thecal cells acting to convert pregnenolone to progesterone before passage to the granulosa cells (*see 1.10*)(**Figure 10**, **Figure 4** [8]). 3β-HSD has two major catalytic activities acting to convert 3β hydroxy-5-ene steroid into 3-keto-4-ene steroids. Two forms of 3β-HSDs exist (type I and II) both capable of using the substrates pregnenolone, 17-OH-pregnenolone and DHEA [61]. Although no variations in basal expression of 3β–HSD has been seen within non-PCOS against PCOS thecal or adrenal samples the possibility of variations in its activity do exist (*see 1.3.2)* [53].

17 β -HSD (17β-hydroxysteroid /17-keto-steroid reductase) is responsible for the final stages of androgen and oestrogen synthesis. It acts in the reversible conversion of 17-keto and 17β-hydroxy groups into androstenedione, DHEA and 17B-estradiol either through oxidation or reduction. (**Figure 10**) [62]. In ovarian steroidogenesis 17β-HSD is found within theca cells and is believed to regulate androgen and oestrogen synthesis through substrate availability and catalysation [63]. Current knowledge 17β-HSD suggests no variation exist in ovarian or adrenal activity of 17 β-HSD [52].

P450arom is expressed within the granulosa of the ovary, catalysing the conversion of androstenedione and testosterone to 17β-estradiol/estrone. Specific P450arom action and location are species specific, with human expression found in the placenta, adipose and ovaries. P450arom expression is only maintained within luteinized granulosa [25, 46]. Increased FSH binding to granulosa membrane receptors in the luteal phase is linked to 17β -estradiol synthesis. It is believed that subsequent rises in intracellular cAMP as a result of this stimulates P450arom expression [9, 46].

1.4 Insulin and steroidogenesis

Insulin is secreted from pancreatic islets of langerhans and is vital in maintaining energy homeostasis through cellular regulation of glucose. In the absence of insulin, body fat is used as an alternate source of energy through lack of glucose uptake. This is seen through the transfer of lipids from adipose to the liver for lipolytic processing [64].

Insulin's ability to stimulate steroidogenesis is unknown. Within the ovaries, it is possible it acts directly in stimulating ovarian androgen secretion and/or augmenting LH stimulated ovarian secretion. Evidence by Munir *et al.*, 2004 supports this and has shown progesterone conversion is activated by insulin action alone within the PI3-K pathway and through the involvement of the insulin receptor [33, 45]. It is also possible insulin may act indirectly in stimulating pulsatile LH release via GnRH with insulin shown to augment GnRH stimulation of LH gene expression. This would therefore suggest that insulin regulation of the reproductive axis maybe effective through its effects on pituitary activity [65]. High levels of circulating insulin and IGF-1 have been shown to lead to decreases in serum hormone binding globulin (SHBG) and insulin like growth factor binding protein 1 (IGFBP-1). Research into examination of physiological levels has suggested that this leads to free IGF in turn stimulating androgen synthesis [66]. As IGF-1 is similar in structure to insulin it stimulates androgen synthesis in a similar manner and is capable of activating the insulin-signalling pathway through PI3-K both through binding to its own receptor as well as IRS. IGF-1 is a polypeptide hormone produced by the liver. Its effects however are twofold less effective in stimulation than insulin with regard to androgen synthesis [67].

SHBG is a glycoprotein produced by the liver that binds to sex hormones (oestrogen, testosterone) to allow their circulation. Free or unbound sex hormones are considered biologically active. Decreased SHBG levels leads to more unbound steroids and therefore more circulating biologically active sex hormones [20].

Insulin action typically precedes the onset of hyperandrogenemia possibly acting alone or with direct stimulation of androgen secretion and/or augmented LH stimulated LH secretion [68]. Alternately, it has been proposed that the indirect action

of insulin may enhance GnRH pulses as increased LH secretion acts to stimulate increased pituitary LH secretion (*see 1.4*) [69].

1.4.1 Ovarian steroidogenesis and the Insulin signalling pathway

Insulin stimulates ovarian androgen through its own receptor [70]. The insulin receptor is a heterotetramer consisting of two ligand-binding α subunits and two tyrosine kinase β subunits (**Figure 11**) [71]. Its action occurs through its extracellular binding to the α-subunits of the insulin receptor (IR) (**Figure 11**). A conformational change within the *β*-chain (IR*β*) activates its tyrosine kinase domain [72] and downstream activation of insulin receptor substrate IRS-1 [73] compartmentalizing the signal through multiple intracellular pathways, including phosphatidylinositol-3-kinase and MAPK.

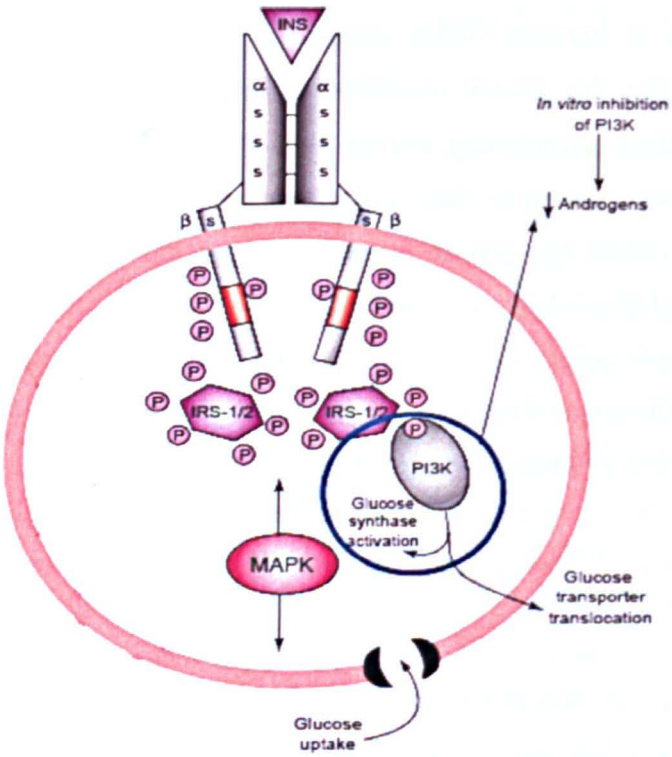

Figure 11. Insulin receptor and activity within human theca cells. (Taken from Diamanti-Kandarakis E et al., 2007).

These intracellular interactions are central in signal transduction allowing actions such as translocation of GLUT4 vesicles to the plasma membrane [71, 74-76].

Phosphorylation of IRβ on serine residues results in inhibition of IRβ tyrosine phosphorylation and can result in interference of insulin action although insulin binding remains unaffected [77, 78].

Studies have shown increased serine phosphorylation of IRβ and inhibition of IRS-1 can be seen as a mechanism of insulin resistance [14]. Furthermore serine phosphorylation of CYP17 can increase androgen biosynthesis (**Figure 12**) [14]. Some women suffering from PCOS have been shown to have increased serine phosphorylation of IRβ and IRS although the mechanism leading to serine phosphorylation is uncertain [79]. The possibility exists as to extracellular serine/threonine kinase causing receptor activation. Alternatively, inhibition of serine/threonine phosphatases may be involved. An example of this is suggested by Guo and Damuni (1993) who have shown that inactivation of protein phosphatase 2A2 (PP2A2) may contribute to increases in intracellular proteins in response to insulin and other mitogens [14].

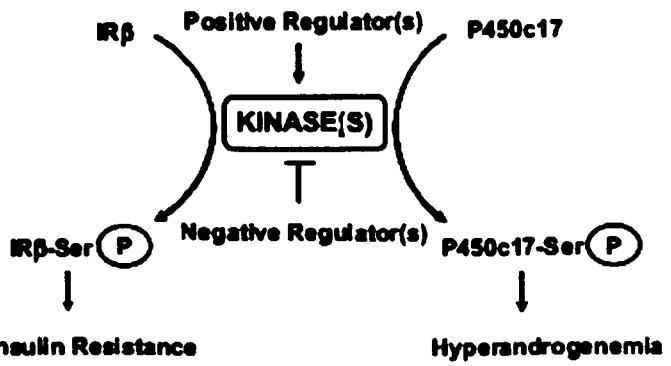

Figure 12 Serine Phosphorylation of IR- β and CYP17. The serine hypothesis proposes serine phosphorylation through dominantly inherited kinase(s) that lead to insulin resistance and increased androgen biosynthesis. (Diagram adapted from Bremer 2008).

It has also been suggested that a gain of function genetic mutation common to both pathways may exist and lead to hyperandrogenemia (**Figure 12**) [80, 81]. Both insulin and IGF-1, a polypeptide hormone similar to insulin and crucial in glucose metabolism, stimulate androgen synthesis via PI3-K/Akt [82]. It is possible that hyperandrogenism and insulin resistance within PCOS is linked through a single serine phosphorylation mechanism (kinase) the result of a post-binding, gain of

function irregularity [14]. Although this theory potentially explains two of the major features of PCOS (insulin resistance and hyperandrogenemia) research has focused entirely on ovarian dysfunction. Furthermore examination of PCOS patients has determined that only a small proportion seem to have increased serine phosphorylation of IRβ [14].

1.4.2 Phosphatidylinositol 3-kinases

Phosphatidylinositol 3-kinases (PI3-kinases or PI3-Ks) are intracellular signal transducer enzymes that phosphorylate the hydroxyl group of phosphatidylinositol inositol rings (**Figure 13**) [83].

Signalling events such as insulin/IGF-1 receptor binding allow production of phosphorylated lipids at cellular membranes subsequently leading to the recruitment and activation of various signalling components.

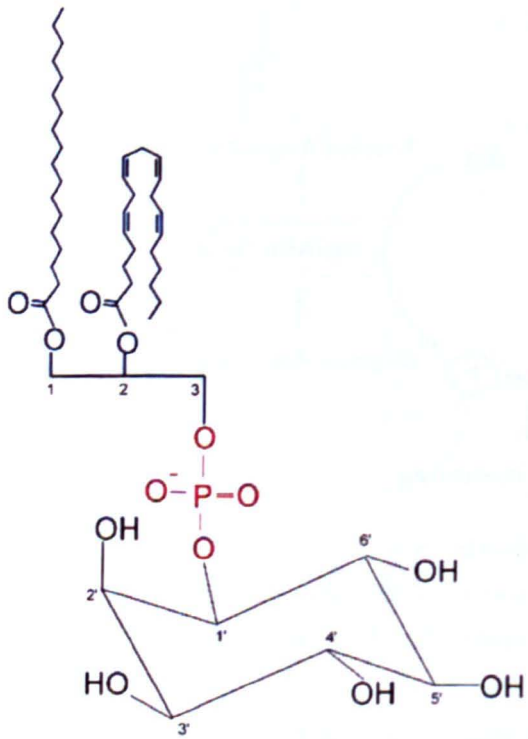

Figure 13. Structure of Phosphatidylinositol. (Taken from Karpova et al., 2006)[84].

These lipid products act as both membrane anchors and regulators, allowing activation of downstream enzymes and subsequent protein substrates [85]. Insulin signalling utilises lipid binding to PH domains of phosphotidylinositide-dependent

protein kinase (PDK) and protein kinase B (PKB/Akt), regulating PKB phosphorylation. This mechanism is used in switch regulation of serine/threonine-specific kinase cascades such as insulin [86].

Phosphorylation of phosphatidylinositol lipids occurs in response to cell stimulation by growth factors and hormones, allowing a coordinated cascade leading to cell growth, migration and/or survival. This process occurs through interactions with intracellular signalling proteins contained within the cytosol that accumulate at membranes during stimulation and prime protein kinase cascades. These include serine-threonine kinases and protein tyrosine kinases along with heterotrimeric guanosine triphosphate (GTP)-binding proteins (G proteins) and utilise specific D-3 phosphorylated phosphoinositide domains allowing tight chemical binding [83, 84, 87].

Protein Kinase B (PKB) also referred to, as Akt exists in three isoforms termed PKBα, PKBL, and PKBQ. It is expressed in all human somatic cells. It can be activated through exposure to insulin allowing the critical link to insulin receptor activation of the PI3-K pathway [88]. Once the insulin receptor is activated PKB is recruited to the plasma membrane through a PI3-K dependent mechanism. This is accompanied by the phosphorylation of Thr^{308} and Ser^{473} on the insulin receptor, activation of IRS-1 and subsequent downstream activation of PKBα [85, 88].

There are multiple forms of PI3-K falling into 3 classes. Typically class Ia are responsible for D-3 phosphoinositide production through growth factor response and other stimuli, with class II and III varying through structural C2 domains located on the C-terminus [89]. Little is known about the functions of class II and III PI3-K and is yet to be fully examined as point of dysfunction within PCOS insulin signalling of steroidogenesis.

Ovarian steroidogenesis utilises this pathway in the biosynthesis of androgen. Research suggests the involvement of class II PI3-K due to the high presence of PI3-K-C2β found within ovarian theca [90].

1.5 Luteinising Hormone and associated androgen biosynthesis.

Intrinsic control of androgen biosynthesis within bovine theca has been shown through LH secretion and contributory GnRH agonists [59]. Research using both human and animal models has also suggested increased androgen synthesis through the individual effects of LH in both non-PCOS and PCOS women [42, 59, 91].

As early as 1958 elevated LH levels were recorded in women with PCOS [92], however experiments by Rebar *et al.,* 1976 showed evidence of wide clinical presentation of PCOS involving normal LH concentrations [93]. The concept of PCOS presenting with LH hypersecretion was later seen as secondary to insulin resistance and not a diagnostic marker [94]. Studies began to focus more on ovarian steroidogenesis when hyperandrogenemia was seen to persist even on suppression of adrenal steroidogenesis through dexamethasome inhibition [95]. These studies support the concept of functional ovarian hyperandrogenism in PCOS [96].

1.6 Polycystic ovarian syndrome

Polycystic ovarian syndrome is the commonest ovarian endocrinopathy affecting up to 10% of women of reproductive age (9) and is associated with significant short and long-term consequences. At least 30% of all those afflicted with the syndrome show all the symptoms [97]. Worldwide prevalence statistics are however only loosely accepted due to variability within diagnostic criteria.

The aetiology and pathogenesis of PCOS is largely unknown. It can be defined as a heterogeneous condition characterized by hyperandrogenic chronic anovulation that has associations with metabolic dysfunction such as peripheral insulin resistance with subsequent hyperinsulinemia [98, 99].

In 1935, Stein and Levinthal first described PCOS as a condition characterised by amenorrhoea, hirsutism, obesity and infertility [100-103]. Their original diagnosis in 1930 was mainly anatomical and was based on the presence of enlarged ovaries with thickened white capsules and multiple sub-capsular cysts. This diagnosis was achieved by surgical excision of 25-40% of the ovarian volume[104, 105].

The base criteria for PCOS diagnosis has always been in dispute, possibly due to the absence of a widely accepted underlying mechanism. The National Institute of Health (NIH)/National Institute of Child Health and Human Development (NICHD) conference in 1990 was the first to define diagnostic criteria for PCOS. The diagnosis of PCOS was based on the presence of classic hyper androgenism (clinical and/or Biochemical), and oligo-anovulation after the exclusion of other androgen excess disorders [106].

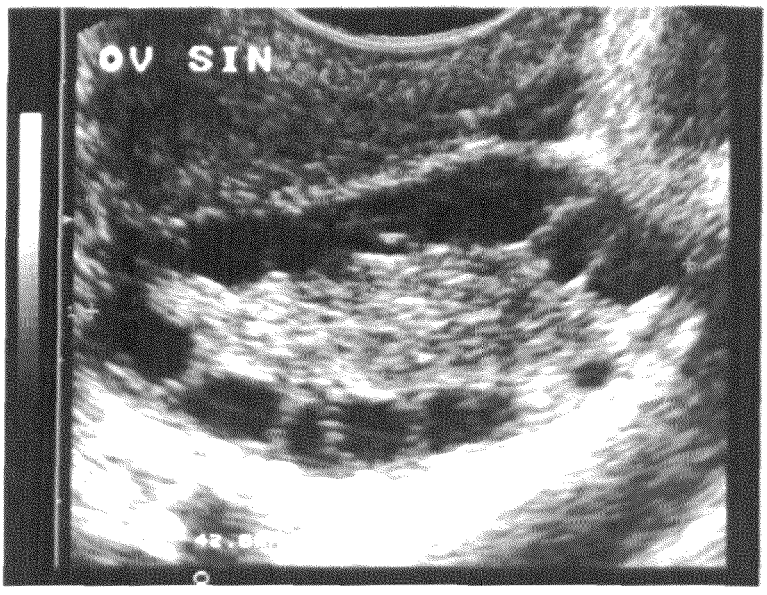

Figure 14. Ultrasound imaging showing polycystic morphology. Trans-vaginal ultrasound showing the morphology of the ovaries when PCOS is suspected. The appearance of a bead like effect often referred to, as a 'pearl necklace' is representative of multiple follicular developments and subsequent arrest before full maturation can occur. (Image adapted from adams *et al.*, 1985)[107].

With the current advances in diagnostics, the diagnostic criteria of PCOS have been revised at the 2003 European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM) consensus workshop conference in Rotterdam [108-111]. According to this consensus, PCOS is now diagnosed by the presence of two of three features including hyper-androgenism, chronic anovulation, and/or polycystic ovaries. The ultrasound criteria of polycystic ovaries have also been revised to include the presence of ≥12 follicles and/or enlarged ovarian volume (≥10ml) (**Figure 14**)[107]. The occurrence of polycystic ovaries alone is not sufficient to allocate a diagnosis of PCOS [111].

PCOS as a syndrome manifests through a varied combination of different clinical features, which usually alter throughout an individual's life from adolescence to the postmenopausal stage. During reproductive age, PCOS women often present with anovulatory infertility oligomenorrhea, hirsutism and obesity. Older women typically suffer obesity, dyslipidemia, impaired glucose tolerance (IGT) and diabetes [112-114].

Chronic menstrual irregularities are considered a dominant characteristic for PCOS [115, 116]. Oligomenorrhea is the commonest menstrual pattern seen in about 75% of

patients with PCOS. About 15% of PCOS women experience amenorrhoea and 10% may have regular cycles [117].

1.6.1 PCOS: Biochemical features:

Hyperandrogenaemia is a common feature in PCOS women affecting about two-thirds of cases. It typically manifests as mild to moderate hirsutism and/or acne [118]. These features usually present in the peripubertal period or during adolescence [119, 120]. Recent studies suggest that hyperandrogenaemia improves with advancing age with subsequent improvement of menstrual pattern [26].

Hyperandrogenaemia in women with PCOS is usually associated with high circulating oestrogen levels. This is possibly due to conversion of excessive peripheral androgens to oestrone especially in overweight women [121]. It has also been suggested that hyper secretion of LH, typical of PCOS, may be associated with increased oestrogen levels [122]. This may lead to continual exposure of the endometrium to excessive unopposed oestrogen without periodic progesterone modulation. This explains the increased incidence and earlier presentation of endometrial hyperplasia and cancer [123]. This association was first noted by Speert in 1949 [124], with limited research into the area to date. Studies by Jackson and Doherty in 1951 are still cited, showing 37% prevalence of endometrial cancer in PCOS patients [125, 126].

The small ovarian follicles found in PCOS have small numbers of granulosa cells with very little or no aromatase activity, which is the enzyme responsible for the conversion of androgens to oestrogen [127]. Those with aromatase activity tend to show high levels of oestrogen and FSH receptors. This suggests possible links to excess androgen formation [128].

Excess circulating androgens in PCOS has been linked to alterations in peripheral steroidogenesis [33, 129]. Such hormonal dysregulation, which represent a key aspect of the pathogenesis of PCOS, have been the focus of research. Several key regulatory enzymes have been extensively investigated including CYP11a, CYP17, 11β-hydroxysteroid dehydrogenase, and 5β-reductase [130-133]. Human and animal

studies have shown evidence of enhanced levels of both CYP17 and 17,20-lyase activity, in ovarian theca cells in PCOS [134-136].

1.6.2 PCOS: Treatment

Typically treatment of PCOS focuses on insulin sensitizing agents including metformin and thiazolidinediones, which aid the reduction of insulin resistance and subsequent hyperinsulineamia. Furthermore metformin has been seen to reduce hyperandrogenemia and although it is unclear of the direct mechanisms behind this, it is understood that it is indirectly linked to both the reductions in circulatory insulin and the possible inhibition of steroidogenic enzymes. Research has shown that metformin stimulates adenosine monophosphate activated protein kinase (AMP-K) a major cellular regulator of lipid and glucose metabolism. In doing so glucose uptake is increased [137]. Additionally metformin has been seen to alleviate glucose toxicity aiding glyceamic control and lipid regulation [138]. In some cases metformin has been shown to inhibit the effects of CYP17 and may therefore explain reductions in androgen levels [139, 140] however contradictory research in human studies has shown that no effect is seen suggesting any reductions in androgen is secondary to the effects metformin has on insulin levels [141].

Similarly to metformin, thiazolidinediones (TZDs) are used to assist insulin sensitivity acting through activation of nuclear peroxisome proliferator activated receptor g (PPARg) which regulates glucose metabolism and fatty acid storage [142]. They also assist in the reduction of androgen levels however this mechanism is also unknown. TZDs such as troglitazone have been shown to directly inhibit the steroidogenic enzymes 3β-HSD / CYP17 however it has been associated with hepatotoxicity [141, 143]. This has also been seen with pioglitazone and rosiglitazone but is only effective at supra-physiological concentrations [141]. The androgen lowering potential of those TZDs in current clinical use may therefore be effective through a more indirect mechanism.

1.7 Steroidogenesis in PCOS: androgen biosynthesis and hyperandrogenemia

In pre menopausal women the ovaries are the main sites of testosterone biosynthesis (*see 1.1.3*) [14]. Additional testosterone steroidogenesis occurs within the adrenal gland and peripheral conversion of other steroids including androstenedione [134, 144].

The developed ovarian follicle structure utilises the thecal and stromal areas surrounding the follicle as the primary site of androgen biosynthesis (**Figure 15**). Following CYP17 activity androstenedione is transported across the thecal membrane for aromatization within the granulosa (**Figure 10**). Failure of aromatization can lead to increases in circulating androgen and subsequent clinical consequences.

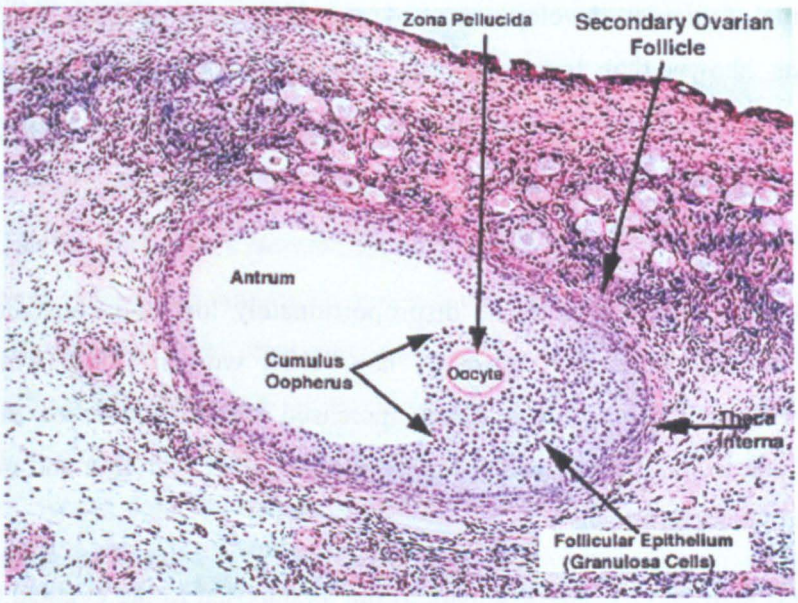

Figure 15. Structure of the mature follicle showing the thecal layer surrounding granulosa. The granulosa encapsulate the oocyte adding to its protective cushion (Picture taken from http://faculty.une.edu/com/abell/histo/secondfollw.jpg).

Typically, excess androgen formation is believed to be associated with PCOS as a result of chronic LH stimulation and subsequent androgen hyper-secretion [144, 145]. However, research has shown abnormal steroid biosynthesis both before and after LH stimulation in human PCOS thecal culture (*see 1.5*) [91].

Examinations into excess PCOS steroid secretion suggests dysregulation maybe prominent at the thecal CYP17 and 17, 20-lyase level [33, 45, 146]. However the effect of LH hypertrophy has not been ruled out. Furthermore, the synergistic effect of insulin excess and increased concentrations of IGF on pre-existing, LH-mediated theca hyper function cannot be excluded [135, 147]. Research has also seen insulin pathway activation through utilization of ovarian insulin receptors, IGF receptors and hybrid receptors, which function through combinations of α and β subunits from both receptors (*see 1.4.1*) [148].

1.8 PCOS and gonadatropin secretion

The two cell two gonadotropin model theory states; both FSH and LH are necessary within normal follicular development and steroidogenesis (**Figure 10**). However research has shown that low dose LH is sufficient for normal development. Furthermore increased serum LH levels have been associated to low fertility rates and spontaneous abortion [149]. This can be seen in relation to PCOS where LH concentrations are typically elevated [69].

FSH levels have been shown to be disproportionately low and constant in PCOS patients when compared with those of non-PCOS women [150]. This may be associated with follicular arrest, although increased inhibin production may also be responsible due to its selective inhibition of pituitary FSH secretion and one primary cause is yet to be determined.

A high steady state of circulatory gonadotropin as opposed to the cyclical fluctuating levels found within normal ovulatory women, has also been hypothesised to result in increased ovarian testosterone, androstenedione, DHEA, DHEAS, 17α-hydroxyprogesterone and estrone [150, 151]. Increased levels of DHEAS have also been found in brothers and sisters of women with PCOS suggesting underlying inheritance [152]. Alternatively the result of hypothalamic pituitary feedback from increased ovarian steroid concentrations may be linked (**Figure 3**, **Figure 5**).

Exaggerated 17-hydroxyprogesterone (17OHP) and androstenedione levels have been seen in response to gonadatrophin releasing hormone agonist (GnRHa) [135] and

human chorionic gonadotrophin (hCG) [153]. These results have suggested the syndrome may have a primary dysregulation of ovarian CYP17 leading to enhanced activity of both 17α- hydroxylase and 17,20-lyase in ovarian.

1.9 Insulin and PCOS

The fundamental abnormality in women with PCOS can be seen through ovarian steroidogenesis, specifically androgens. It is therefore hypothesised that a defect in the steroid secreting theca cell is involved [45, 154]. Thecal cells express LH receptors, IRs, lipoprotein receptors both HDLs and LDLs, StAR protein, P450scc, 3B-HSD and CYP17, all vital to ovarian steroidogenesis [99]. Examination into the variations in steroidogenic within PCOS theca cell has yet to pinpoint any dysfunction in expression or activity and insulin.

Hyperinsulinemia has been associated with hyper-androgenemia through *in vitro* studies of cultured PCOS theca, where it has been suggested that insulin increases progesterone and androstenedione secretion when compared to normal theca cells [155, 156]. It has been demonstrated that insulin acts via its own receptor within PCOS cultures, increasing androgen concentrations [157-159]. Human PCOS and non-PCOS theca have been shown to increase testosterone secretion through the individual effects of insulin and studies have also shown increased androstenedione secretion in porcine theca under combined stimulation of insulin and LH [9, 157, 160]. Furthermore, recent data has shown a role for insulin and LH in the expression of genes encoding StAR and CYP17 in porcine cellular models [42]. Research has yet to fully support the individual effects of insulin stimulated androgen within human models and reports are contradictory with later studies using monolayer theca cultures showing lack of increasing insulin stimulated androstenedione secretion in non-PCOS samples [161, 162].

Many theories exist toward the etiology of hyper androgenemia within PCOS [14, 33, 159]. Very few approaches however, focus on steroidogenic regulation utilising isolated thecal cells. Our study employs isolated thecal cultures with close regulation of the influential factors LH and insulin. In doing so, we have analysed androgen synthesis through activation and expression of steroidogenic enzymes CYP17 within

both normal and polycystic ovaries. This allowed us to examine whether protein concentrations vary in relation to the existence of possible abnormalities in the insulin-signalling pathway theca [135, 153].

1.9.1 Insulin resistance in PCOS

Abdominal obesity (BMI/WHR) is positively associated with increased risk of abnormal glucose tolerance with subjects showing marked increases in glucose levels [163, 164]. The strong correlation between increases in BMI and gradual increase in Glucose Tolerance Test (GTT) severity within PCOS patients suggests further deterioration of glucose metabolism when compared to normal patients [163].

Obese PCOS women tend to be more insulin resistant than obese non-PCOS and obese non-PCOS have been shown to be more resistant than lean PCOS patients [165]. This suggests that the insulin resistance present in association with PCOS is independent of obesity and that the co-existence of obesity is an additional albeit contributory factor. The Rotterdam consensus panel subsequently recommends glucose tolerance tests for obese PCOS patients recognizing this association [166].

Research has shown the pathogenic mechanisms of impaired insulin action are complex and multifactorial supporting the heterogeneity of PCOS. Insulin resistance leads to the prevalence of gestational (GDM) and type 2 diabetes (T2DM) and is common in PCOS. This can subsequently lead to inadequate insulin secretion, β-cell exhaustion, insulinopenia and the development of T2D [167]. One theory is that the insulin resistance found in PCOS consequently amplifies the demand for insulin secretion via pancreatic beta-cells [168]. This in turn can lead to beta cell dysfunction and development of T2D or GDM [169]. It has also been shown that beta cell dysfunction is prevalent within PCOS patients and that a hereditary component may exist [170]. However with all this research it is still uncertain whether beta cell dysfunction precedes the development of insulin resistance.

Pathophysiology and molecular defects within PCOS are typically associated with defects in insulin action/secretion leading to profound insulin resistance and pancreatic β-cells dysfunction [166, 167, 171]. Insulin resistance is the main

constituent of the metabolic syndrome with 70% of PCOS patients suffering irrespective of BMI [113]. Current concepts of insulin signalling defects point to an element of tissue specificity [33]. Insulin auto phosphorylation within adipocytes has been seen to be decreased by 30% in PCOS patients [172]. It has also been seen that serine phosphorylation in fibroblasts is increased by 50% and that insulin substrate 1 association with PI3-K activity in muscle is lower in comparison to normal cells (IRS1 associated P13-K)[33].

Metabolic abnormalities seem to be prevalent with the PCOS diagnosis. These can include changes in cholesterol and triglycerides, fibrinolysis and plasminogen activation [173, 174]. The occurrence of diabetes mellitus and impaired glucose tolerance within PCOS sufferers with worldwide prevalence of around 30 – 40% [175]. Insulin resistance is defined as an impaired metabolic response to insulin and puts those suffering at an increased risk of glucose intolerance with 10% of PCOS patients under 40 developing T2DM [176, 177]. The risk of diabetes mellitus within PCOS patients is therefore believed to be linked to obesity; however studies show that the links to PCOS and the underlying insulin resistance may also contribute [112]. Although insulin resistance is believed to be independent of obesity, PCOS studies have shown that obesity may increase insulin resistance and subsequent hyperinsulinemia and increased insulin resistance has been shown in women with central fat distribution [178]. Research has also shown that regardless of BMI, PCOS women present with central obesity and gynoid distribution [179]. Furthermore studies have reported that excess visceral fat and peripheral fat distribution is associated with metabolic disturbances, insulin resistance T2DM, dyslipidemia hypertension and CVD [178, 180, 181]. It is therefore possible that the increase in obesity plays a crucial part in the occurrence of PCOS in susceptible individuals and reproductive disturbances are seen to be more frequent in obese women than normal-weight women independent of PCOS [182].

Although insulin resistance is seen in most women with PCOS [183], the effects of insulin action on such processes as mitogenesis and steroidogenesis, have been shown to be normal in PCOS patients with the effects on glucose and lipid metabolism

usually seen to be impaired [142]. This as a result affects whole body insulin sensitivity in PCOS [41, 159, 184].

The exact mechanism behind insulin resistance in PCOS is unknown. Typically insulin resistance is the result of inherited or acquired influences such as mutations in insulin receptor, glucose transportation or signalling protein [183]. Investigations into these areas in target tissues including ovarian and adipose within PCOS patients has yet to pinpoint any defect. Studies into adipocyte of PCOS patients have determine decreases in maximal rates of glucose transportation suggesting post binding defects in signalling pathways [172]. A significant reduction has also been seen within glucose transporter 4 (GLUT4) and both these defects are seen to be independent of glucose intolerance, obesity and levels of circulatory sex hormones. This may therefore be reflective of an intrinsic PCOS related defect [185].

1.10 Obesity and PCOS

The association of obesity and PCOS is well established with prevalence recorded as high as 75% dependent on country or ethnicity [174]. It is also accepted that PCOS patients tend to have a distinct gynoid body fat distribution independent of BMI with central fat accumulation known to be related to metabolic disturbance (see 1.14.). Infertility and menstrual irregularities are seen to be more frequent in obese patients both non-PCOS and PCOS and there is no doubt in the ability of obesity to aggravate PCOS symptoms. Often weight reduction is seen as a therapeutic target that has been shown to alleviate reproductive dysfunction in PCOS patients reducing ovarian volume and follicle count [186, 187]. Reduction in weight in PCOS patients has also shown improved fertility rates and menstrual patterns [187, 188] Furthermore insulin resistance, which is seen as central to PCOS, is also aggravated by obesity and again studies have shown enhanced insulin sensitivity as a result of weight loss [188, 189].

Hyperandrogenemia is seen as diagnostic feature of PCOS and with reduced plasma SHBG levels typically found in obese PCOS patients leading to increased free androgen levels further association may be seen. Androgen has also been shown to effect overall body fat distribution favouring centralized deposition and women with centralized obesity have been shown to produce more testosterone than those with

peripheral obesity [188, 190, 191]. All this evidence points to an underlying involvement of adipose / adipocytes in the occurrence of PCOS.

1.11 Adipose, adipocytes and adipokines

Up until the discovery of leptin in 1994 by Zhang *et al.*, adipose was regarded as means of storage and mobilization of energy [192]. Since its communicative ability was determined via its effect on the hypothalamic neuropeptide-Y (NPY) and its subsequent energy balance regulation adipose tissue has been regarded as a more complex endocrine organ of which its full ability is yet to be understood [193]. Research has shown it is capable of secreting many varied bioactive peptides and proteins known as adipokines (*see 7.4*). These have been shown to act locally with both autocrine and paracrine action, as well as throughout other areas of the body allowing cross talk with other physiological systems [194]. Its importance in acting on whole body homeostasis and influencing physiological processes has become more understood with its involvement in inflammation, blood pressure regulation, lipid metabolism, insulin sensitivity and coagulation [195]. It therefore seems that irregularities within adipokine secretion may lead to physiological dysfunction and it is believed that this may lead to influences in adrenal and ovarian function including steroidogenesis and fertility [196, 197]. This is supported by the importance leptin has in physiological process including energy homeostasis and its influence in gonadal and reproductive function. Whereby leptin has been seen to stimulate the hypothalamus and pituitary gland [198].

1.12 Adipokines in PCOS

Adipokines have been shown to play important roles in female metabolism. Leptin has been seen to play a role within the female reproductive organs with target tissues including placenta mammary glands and endometrium subsequently influencing physiological processes including menstruation and pregnancy [198, 199]. Although leptin levels have not shown to be altered in weight and age matched PCOS patients [200] examination into the effects these levels may have on reproductive processes in PCOS patients is yet to be fully understood. Examination of additional adipokine secretion in PCOS and non-PCOS women, have shown disturbances may exist.

Research by Mahde (2009) into 60 PCOS patients showed a significant increase in serum interleukin-6 (IL-6), interleukin-8 (IL-8), resistin, leptin RBP-4 and tumor necrosis factor-a (TNF-a) levels [201]. More specifically Mahde examined the relationship between PCOS and omentin-1. With omentin-1 seen to be reduced in obese patients. With its involvement in increasing insulin signal transduction and enhancing insulin-stimulated glucose transportation omentin-1 is believed to be linked to insulin resistance [202]. Mahde therefore concluded that this adipokine may play a paracrine/endocrine role in modulating insulin sensitivity with particular regard to PCOS [201]. This seems feasible with studies into adiponectin showing an inverse relationship between body weight insulin resistance and T2DM [203, 204]. Furthermore recent studies have shown PCOS patients to have lower levels of adiponectin to that of non-PCOS patients when age and BMI matched [205].

1.13 Adipose, androgens and PCOS

As discussed in section 1.7 androgen is believed to play an important role in the underlying cause of PCOS. With the association of obesity to PCOS (*see 1.10)* and the understanding that androgen imbalances occur as a result of weight gain independent of PCOS [174, 206], The role of adipocytes within endocrine imbalance is of great interest to the condition.

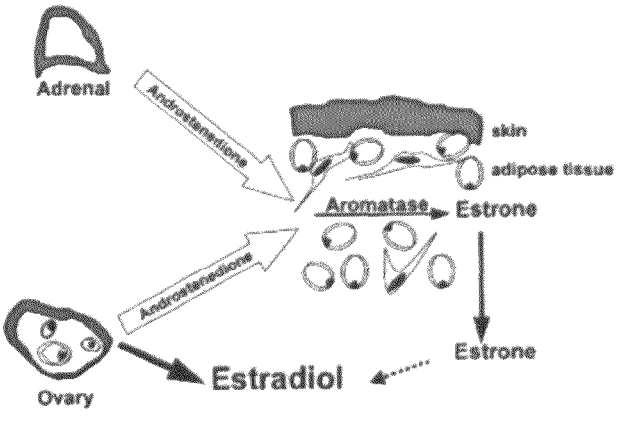

Figure 16. Adipocyte and Fibroblast sex hormone synthesis. Current understanding of the role of adipocytes and fibroblasts in the synthesis of sex hormones through activation and deactivation of adrenal/ovarian precursors.

The role of adipose as an endocrine organ includes the activation and deactivation of sex steroids (*see* **Figure 16**). Furthermore adipocytes have been seen to synthesize the steroid hormone oestrogen acting as the main physiological supply in post menopausal women [207]. It is understood that the main source of female androgens are from adrenal and ovarian synthesis, however research has shown that local adipocyte concentrations are significantly higher than levels found in systemic circulations [203]. This fact, coupled with the presence of steroidogenic enzymes key to androgen biosynthesis found in adipocytes tissue including aromatase, 3β-hydroxysteroid dehydrogenase (HSD) type 1 [191] 11β-hydroxysteroid dehydrogenase type 1 and 2 [208] 5α-reductase [209] and 17β-HSD types 2,3 and 5 [210] suggest adipose may play a vital function in more than androgen activation and processing. Furthermore with androgens shown to significantly increase with respect to weight gain this may be seen as a possible mechanism in the symptomatic hyper-androgenemia found in PCOS.

2.0 HYPOTHESIS

2.1 Primary hypothesis:

The specific hypothesis to be tested is that adipocyte and theca cells in women with PCOS have an intrinsic defect within the insulin-signalling pathway resulting in increased sensitivity to insulin and increased androgen synthesis.

2.2 Objectives

- To determine whether excess androgen production in women with polycystic ovarian syndrome (PCOS) is due to a primary adipocyte/theca defect or secondary to hyperinsulinaemia and/or LH-hypersecretion.
- To determine the insulin-signalling pathway involved in the stimulatory effects of insulin on theca cells in PCOS.
- To investigate the possibility of an alternate endocrine function within adipocytes and its association to symptomatic hyper-androgenaemia in PCOS.
- To investigate the possibility of variations / dysfunction between non-PCOS and PCOS adipocytes.

3.0 MATERIALS AND METHODS

3.1 Patient demographic and recruitment

All samples were collected from the Department of Obstetrics and Gynaecology at the Royal Derby Hospital. The study and associated techniques for collection/processing, were approved by the Derbyshire Ethics Committee (Study reference: RD-5103-015-08).

The details of the study were discussed with each patient by the researcher or clinician. All patients were fully informed verbally and also given patient information sheets with the details of the research and procedure required for tissue collection (see appendix). All participating patients gave written consent in triplicate (patient/PI/medical records) and assigned study numbers in order to provide anonymity.

3.1.1 Adipose tissue collection

Adipose tissue biopsies (~5g) were taken from subcutaneous adipose tissue of the abdominal wall of participating women either during a planned surgical procedure or under local anaesthesia in the outpatient clinic (for women not undergoing surgery). Two groups were selected; normal women (control group) and women with PCOS. Data was summarised in **Table 2**.

The control group (non-PCOS) consisted of women with regular menstrual cycles (28-32 day cycle), normal serum levels of androgen (0.2-2.9nmol/l), luteinizing hormone (luteal phase 1-11nmol/l) and fasting insulin (17.8-173pmol/l) with data summarized in **Table 2**. All women were of childbearing age, ranging from 20-45 years with a BMI $\leq$ 35 kg/m^2.

The women within the PCOS group were diagnosed according to the 2003 Rotterdam ESHRE/ASRM consensus with two of the required criteria from:

- Chronic oligo- or anovulation
- Clinical and/or biochemical evidence of hyperandrogenism (biochemical: testosterone $\geq$ 2.5nmol/l or free androgen index $\geq$ 5)
- ultrasound appearance of polycystic ovaries.

Diagnostic criteria based on Rotterdam revised consensus and not clinical management debate which argues to include subcategories of patients by the previous NIH criteria which does not include signs of polycystic ovaries [108, 211, 212].

Exclusion criteria included for both adipose and ovarian sample collection;

- Metabolic or endocrine disease, such as diabetes mellitus and thyroid disease.
- Bilateral ovarian disease (excluding simple/functional ovarian cysts). In women with a unilateral disease, the biopsy was to be taken from the non-diseased ovary.
- Concurrent treatment with: hormonal therapy such as hormonal contraception, progestogen therapy, thyroxin hormone or corticosteroids.
- Metformin.
- Cholesterol lowering agents.
- Patients unable to give signed informed consent e.g. those with mental incapacity.
- Patients unable to understand verbal or written information in English.

Women with a diagnosis of PCOS were screened for the above inclusion/exclusion criteria for the study group by taking a medical history, performing a clinical examination, arranging an ultrasound scan and a blood test. All these screening measures were part of the routine management of women with PCOS. Patients who were suitable for the study were given a Patient Information Sheet (PIS) and filled out the relevant consent form.

Parameter	Normal (n=17)	PCOS (n=10)
Age (yrs)	33.8 (24-44)	30.5 (25-38)
Body Mass Index (kg/m^2)	26.4 (17.6-33.1)	32.3 (19.8-37.65)
Insulin (pmol/l)	26.0 ± 5.8	118.6 ± 31.8
Testosterone (nmol/l)	1.4 ± 0.2	2.4 ± 0.4
Luteinising Hormone (nmol/l)	6.7 ± 1.4	29.9 ± 11.2

Table 2. Patient demographic of cultured samples. Data summarised as mean (range) / SEM (±).

The age range of our samples allowed them to be closely matched between groups with all subject. All samples used were chosen from subjects that were not midcycle and therefore either in the luteal or follicular phase of their menstrual cycle allowing normalization, with only 4 of the PCOS women having oligo-ovulation. Those subjects midcycle have increased LH levels typically found in PCOS women (>96nmol/l). Biochemical variations between the groups existed with significant increases in testosterone (>2.9nmol/l), insulin (>170pmol/l) and LH levels symptomatic of PCOS women. Recent studies have shown that DHEAS is less reliable than testosterone with only 5% of hirsute patients examined showing elevated levels [213]. For this reason we measured free testosterone levels.

3.1.2 Ovarian tissue collection

Following patient consent a 1-2cm biopsy was taken from each ovary either during surgery (hysterectomy) or on removal of the ovary (oophorectomy) through careful scalpel incision. Both control and PCOS samples were collected using the same exclusion criteria as mentioned in 2.1.1. Samples were then immediately transported to the laboratory in collection media consisting of Hanks Balanced Salt Solution (HBSS) containing, 1% penicillin (100U/ml), 100U/ml streptomycin (10000U/ml), HEPES (15mM). Samples not used for primary culture were snap frozen and stored at -80°C for later use in western blotting.

3.2 Mammalian cell cultures

3.2.1 3T3-L1 pre-adipocytes

Initial adipocyte experiments utilized an established animal cell line (3T3-L1) whilst awaiting ethical approval for use of human biopsies. These were kindly supplied by Dr Saoirse O'Sullivan (Division of Vascular Medicine, School of Graduate Entry Medicine & Health, University of Nottingham). This cell line was stored frozen in DMSO/serum and taken from nitrogen storage at passage 20, quickly thawed by immersion in a water bath at 37°C then seeded in T25 flasks in DMEM media with high glucose 4500mg/l (v/v) and 10% fetal bovine serum (FBS). The composition of all the various buffers used throughout this project including suppliers and their addresses, have been listed in the appendices (see appendix).

Following 24 hrs incubation at 37°C, culture media was refreshed to remove DMSO. Culture medium was changed every 48hrs thereafter until confluence was reached at around day 7 and the cells were passaged further in a T75 flask to increase cell number.

3.2.1.1 3T3-L1 pre-adipocyte differentiation

Preadipocytes were seeded in 96 well plates in DMEM high glucose (4500g/l) + 10% FBS media. These were grown to 80-90% confluence (*see* **Figure 17**) and the media (termed initiation media) consisting of high glucose DMEM (high glucose), 10% FBS, 2mM L-glutamine, 1% penicillin/streptomycin, 0.5mM isobutylene-1-methylxanthine, 1μM insulin, 1μM dexamethasone. Following 3 days incubation at 37°C, initiation media was removed and differentiation media added consisting of high glucose DMEM (high glucose), 2mM L-glutamine, 1% penicillin/streptomycin 50nM insulin, 1μM dexamethasone. Media was replenished for fresh differentiation media every 2 days until lipid droplet formation was observed, typically at around 28 days (*see* **Figure 18**). Differentiation was then validated using Oil Red O staining (*see 3.2.2.1*).

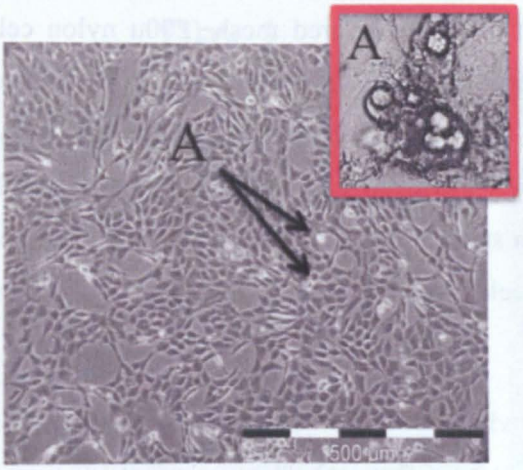

Figure 17. 3T3-L1 Preadipocytes 7 days following dispersal maintained at around 90% confluence. Preadipocytes appear as phase-dark, polygonal cells. (**A**) Inset shows preadipocytes beginning to differentiate.

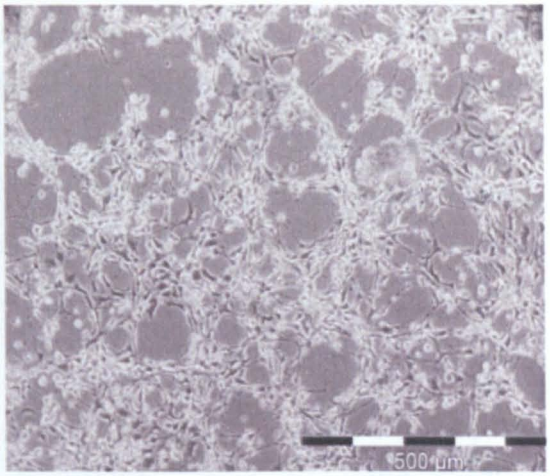

Figure 18. 3T3-L1 Adipocytes. Preadipocytes following 4 weeks of routine media changes occurring every 3 days with media consisting of insulin, IBMX and dexamethasone.

3.2.2 Preparation of subcutaneous human adipose primary cultures

Approximately 5g of subcutaneous adipocyte tissue (**Figure 19**) collected from theatre or under local anaesthesia in the outpatient clinic was immediately transported to the laboratory in a collection media consisting of HBSS, 1% penicillin (100U/ml), streptomycin (100U/ml), HEPES (15mM).

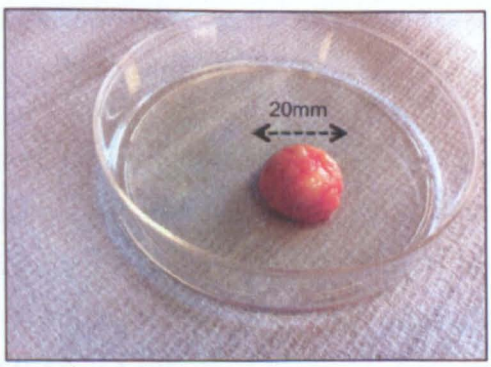

Figure 19. Subcutaneous adipose biopsy. Subcutaneous adipose tissue biopsy (~5g) taken from consenting patient within the outpatient clinic under local anaesthesia.

Adipose biopsies in HBSS were minced with a scalpel blade and tissue enzymatically digested with collagenase (1mg/ml), prepared in HBSS, for 1 h at 37°C. Thereafter,

the digested material was filtered through a double-layered mesh (200u nylon cell micro sieves). Following separation through the mesh the isolated cells were then centrifuged at 250x g for 10 mins (x2) at room temperature in HBBS and the supernatant was discarded. Finally the pellet was re suspended in DMEM (high glucose), 2mM L-glutamine, 1% penicillin/streptomycin 10% FBS and incubated at 37°C 5% O^2/95% CO^2 until sufficient yield typically used at passage 2 for the differentiation procedure.

All passages followed the same protocol whereby culture media was removed and cells carefully washed using HBSS (without $MgCO_2$ or $CaCl_2$). Trypsin/EDTA was then added and incubated for 2 mins at 37°C 5% O^2/95% CO^2 until rounding of the cells was seen under a microscope. Media containing 10% FBS was then added to deactivate the trypsin/EDTA and plates were then gently tapped to remove any remaining adherence. Cells were then spun at 250 x g for 5 mins in relevant media dependent on the cells, and supernatant removed before plating in the relevant media.

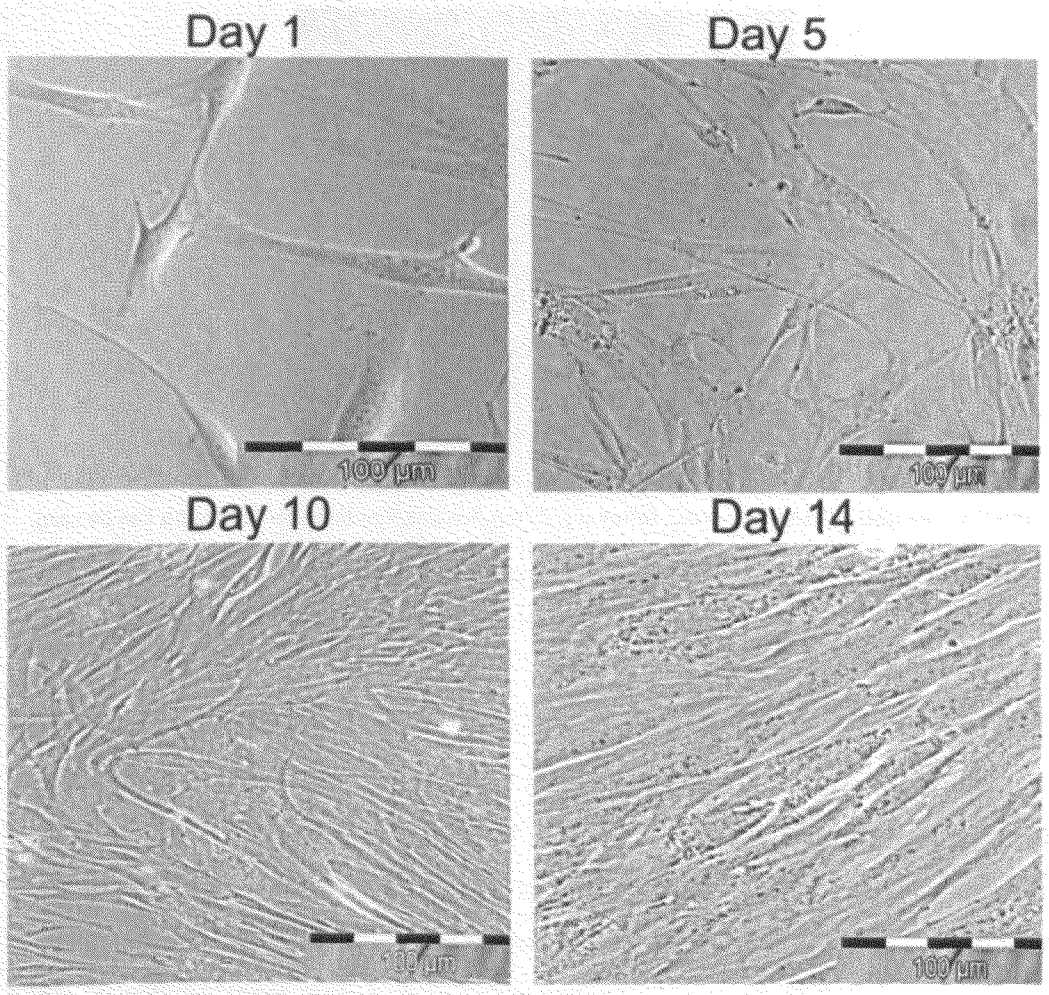

Figure 20. Human preadipocytes 14 days *in vitro*. Preadipocytes maintained in DMEM+10% serum. Morphologically the cells are elongated at day 1. Day 5 shows the cells increasing in number. By day 10, the cells begin to form a tight matrix until day 14 when 80-90% confluence gives a striated effect and firm adherence of cells to the base of the flask.

Preadipocytes (see **Figure 20**) were cultured in DMEM high glucose + 10% FBS to 80-90%. At day 14, media was removed and changed for differentiation medium containing 50nM insulin, 100nM dexamethasone, 0.25mM 3-isobutyl-1-methylxanthine for the first 3 days. The medium was then replaced with maintenance media consisting of DMEM and 10% fetal bovine serum, 50nM insulin, and 100nM dexamethasone, and changed every 2 days until accumulation of lipid droplets within cells (D14-21) see **Figure 21**. Lipid droplets were assessed by staining fixed cells with Oil Red O (*see 3.2.2.1*).

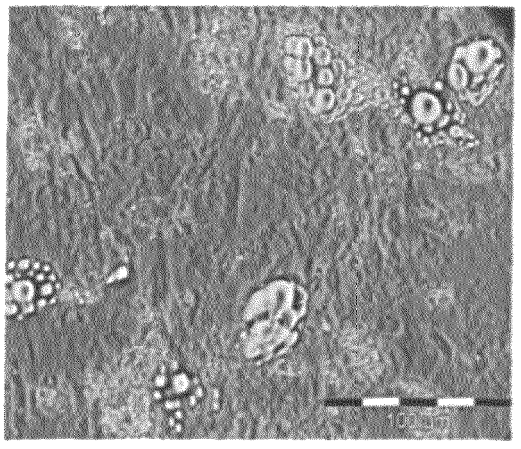

Figure 21. Non-PCOS control preadipocytes following differentiation with insulin, and dexamethasone and IBMX. 8-9 weeks following differentiation, lipid droplet formation can be seen.

3.2.2.1 Adipocyte Oil Red O staining

Following 8 weeks of routine media changes using differentiation media, cultures were stained using the Oil Red O technique to determine lipid droplet formation. Cells, grown in 24-well plates, were carefully washed in HBSS and fixed with 4% paraformaldehyde (PFA). 100ml (PBS) was measured and added to a conical flask containing 4g of paraformaldehyde. This was covered with parafilm and transfer to a fume hood to be left on as automatic shaker for 30 mins thoroughly shaken at 37°C until clear. This was then allowed to cool before storage at -80°C.

Following fixation (*see* 3.5) PFA was removed and cells left to air-dry completely. A working solution of Oil Red O consisting of 6 parts Oil Red O and 4 parts dH_2O was mixed in a test tube and filtered through 0.2µm syringe filter in order to remove particulate matter. 1 ml of this solution was then carefully added to each well (1ml per well/6 well plate) and left for 10 mins at room temperature before removal and careful washing (x4) with dH_2O. Cell staining was then observed with a microscope see **Figure 22.**

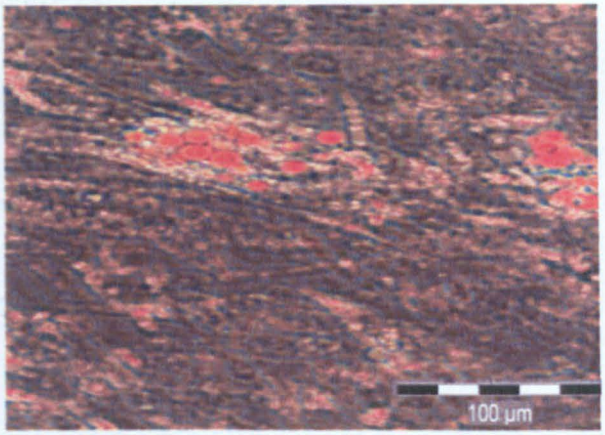

Figure 22. 4 weeks differentiation of non-PCOS adipocytes stained with Oil Red O. Lipid droplets are clearly apparent as a pink to deep red colour.

Differentiation of adipocytes (*also used with theca cultures*) was measured using a method adapted from the Oil Red O staining (adapted from the protocol by L. Jenkarova (2005)). PCOS and non-PCOS cell cultures were grown to confluence and differentiated (*see 3.2.3*). Adipocytes were then stained at time courses (1, 3, 6 and 9 weeks). The Oil Red O was eluted by adding 100% isopropanol and incubated for 10 mins. The solution; including isopropanol and Oil Red O, was transferred to a 96 well plate to be read via spectrophotometry (OD 500nm). All results were examined against preadipocytes grown under non-differentiation conditions and blanks.

3.2.3 Ovarian theca culture.

All women scheduled for any abdominal or laparoscopic gynaecological surgery, such as laparoscopic ovarian diathermy (for women with PCOS), laparoscopic sterilisation or hysterectomies were considered for the study under the inclusion/exclusion criteria (*see 3.1.1*). During surgery, one small cortical biopsy was taken from the anti-mesenteric surface of the ovary by one of the investigators (Mr Amer or Professor Shaw). Ovarian tissue was then collected into HEPES-buffered Minimum Essential Medium for immediate transfer to the laboratory. Samples were anonymised and coded immediately

Ovarian specimens were placed in a 6mm petri dish and washed with HBSS immediately to remove blood. Individual follicles were identified under a dissecting microscope then dissected from the ovarian stroma using fine forceps, taking extreme care not to rupture the follicle. For cell dispersal, viable un-ruptured follicles were chosen based on their size (>4mm) so as to allow dissection without the aid of a microscope. Typical biopsies contained 1-2 follicles no larger than 4mm, which required careful processing in order to obtain viable cells.

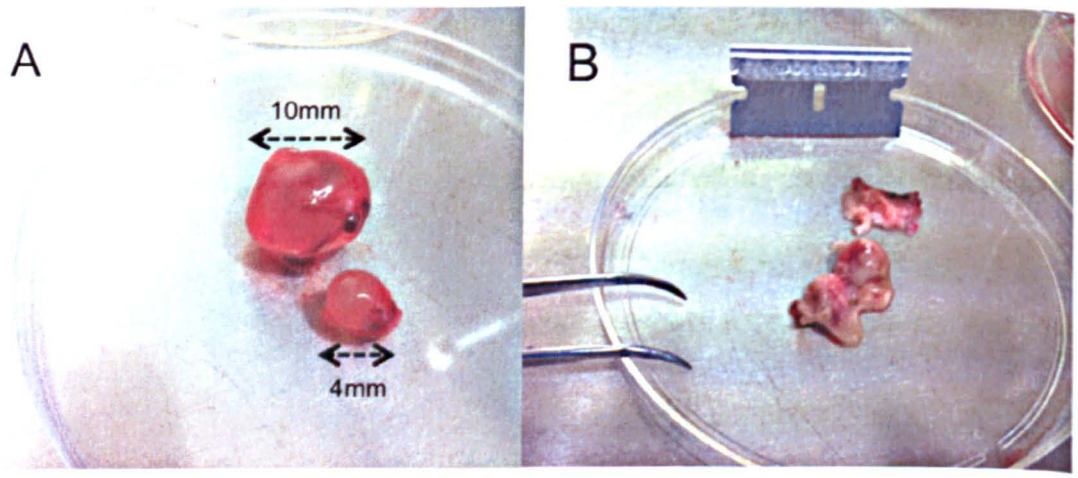

Figure 23. Ovarian follicles. (A) Follicles removed from ovarian stroma through careful dissection. (B) Once follicular fluid is removed the follicles are opened up to show the thecal sheets.

Follicles were dissected carefully minimising contamination from non-follicular tissue (*see* **Figure 23**). The granulosa were removed through aspiration by careful insertion of a 25Gx1mm needle (25ml syringe). Aspirated follicular fluid was centrifuged 250

x g for 10 mins and pellet/granulosa cells re-suspended in DMEM/F12 with 10% FBS for culturing see **Figure 24**.

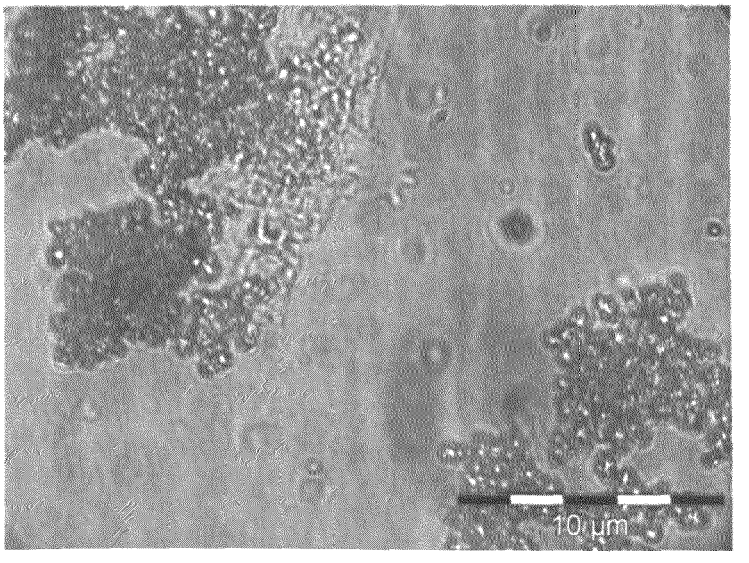

Figure 24. Human granulosa cells following 24hrs plating passage 1.

The deflated follicle was opened and spread in fresh HBSS allowing thecal sheets to be slowly separated from the inner lining through peeling with fine forceps. The collected theca were carefully flushed through a 1ml syringe containing enzyme dissociation solution consisting of collagenase I 1mg/ml, hyaluronidase 0.5mg/ml and DNase I 15kunits (see appendix) and then incubated for 10min at 37°C. The cells were then disaggregated with gentle pipette flushing in HBSS and washed twice with centrifugation at 250 x g for 10 min at room temperature [45, 67, 130, 214-216].

The theca were re-suspended in theca media composed of DMEM/F12 modified, with 15mM HEPES, 2.5mM L-Glutamine, pyrodixine HCl, 55mg/l sodium pyruvate, 1% penicillin (100U/ml), streptomycin (100U/ml), selenium (20nM), insulin (20nM), FBS 10% (v/v), 2% UltroSer G serum [214, 217]. Theca cells were adjusted to a final cell count of 10^4 cells/ml and cultured for 7-10 days to confluence and passaged as required through trypsinisation.

3.3 Hormone assays

Cells (adipocyte/theca) (passage 3) were maintained in serum-free conditions for up to three days to eliminate any contribution from hormones in serum and used in hormone assays.

- The effects of insulin were tested by incubating cells with insulin (10-100ng/ml).
- The effects of LH were tested by incubating cells with LH (10ng/ml).
- The effects of insulin and LH were tested by incubating cells with insulin (10-100ng/ml) +10ng/ml LH.
- All treatments were also tested under PI3-K inhibition using LY294002 treatment (*see* **Figure 25** */ hypothesis 2.1.*).

Following passage of primary cultures cells (adipocyte/theca) the viability of the theca cells was assessed using trypan blue exclusion. A solution of 10µl of media and 10µl trypan blue was added to a 1ml solution of media/cell suspension. A haemocytometer was used to examine 10µl of the cell suspension solution under a microscope and the % viability was calculated using haemocytometer (% viable cells = no. of unstained/total cell count). $2x10^5$ cells were then plated in the appropriate serum free theca/adipocyte media (*see method 3.2.1/3.2.3*) + treatments (*see* **Figure 25**) and harvested dependent on the required time course (24, 48 or 96hrs).

At the relevant time point (*see* **Figure 25**) cells were harvested and conditioned media removed which was stored at -80°C for later examination of hormone levels.

In order to investigate cell viability we monitored proliferation and took regular cell counts using trypan blue (*see 3.4*). Cells were carefully washed with HBSS and 100µl of trypsin/EDTA added for 2 minutes before cell suspension removed from the well. Cells were counted using a haemocytometer and further washed in HBSS. Cells were then centrifuged at 250 x g for 10mins and supernatant discarded. The cells were then lysed for use in western blotting (*see 3.6*).

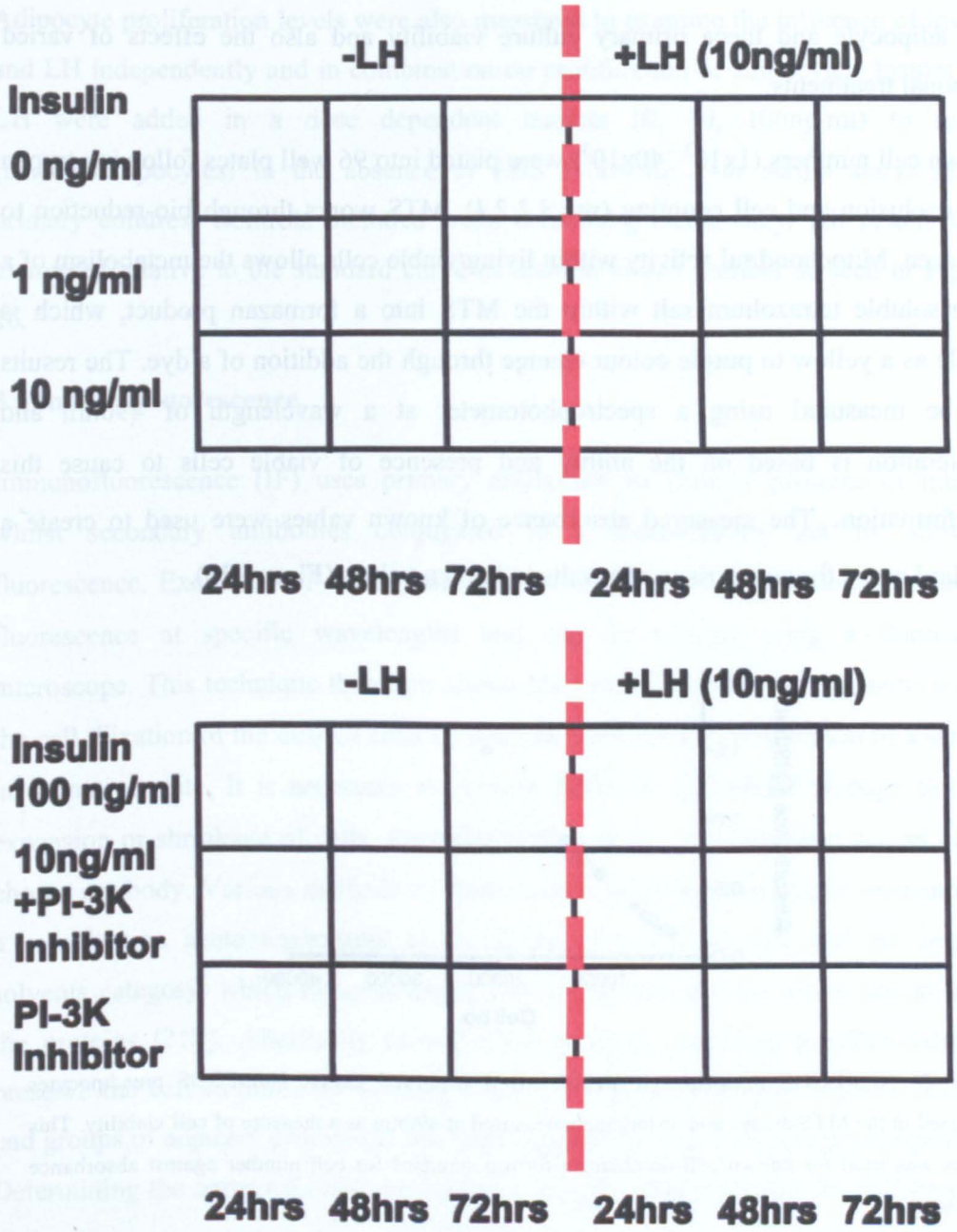

Figure 25. Theca/Adipocyte cell treatments. Cells were plated $2x10^5$ in varied insulin/LH treatments and harvested at varied time courses (24, 48 and 96 hours).

3.4 Proliferation Assays

Proliferation assays of theca cells and adipocytes were undertaken in addition to the hormone assays. This was measured using the MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and colorimetric assessment through spectrophotometry (490nm OD). This allowed examination of

both adipocyte and theca primary culture viability and also the effects of varied hormonal treatments.

Known cell numbers ($1x10^3$ - $40x10^3$) were plated into 96 well plates following trypan blue exclusion and cell counting (*see 3.2.2.1*). MTS works through bio-reduction to formazen. Mitochondrial activity within living/viable cells allows the metabolism of a water-soluble tetrazolium salt within the MTS into a formazan product, which is visible as a yellow to purple colour change through the addition of a dye. The results can be measured using a spectrophotometer at a wavelength of 490nm and proliferation is based on the ability and presence of viable cells to cause this transformation. The measured absorbance of known values were used to create a standard curve for comparison of results to known values (**Figure 26**).

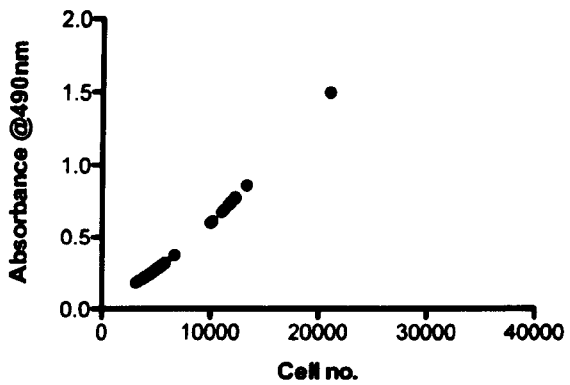

Figure 26 Non-PCOS preadipocyte proliferation standard curve. Non-PCOS preadipocytes were used in the MTS assays and absorbance measured at 490nm as a measure of cell viability. This process was used for known cell numbers to form a standard for cell number against absorbance (@490nm).

For assessment of proliferation, non-PCOS/PCOS preadipocyte and theca proliferation assays were established to examine variations across basal levels. Cells taken from primary cultures between passages 3-4 were plated at a density of $5x10^3$ (n=5). At consistent time intervals (24, 48, 96, 120 and 144hrs) 20µl of MTS was added to each well and incubated at 37°C for 1 hour before measuring absorbance at 490nm. A standard curve was created using known cell numbers and their corresponding absorbance. All results were examined using the standard curve **Figure 26.**

Adipocyte proliferation levels were also measured to examine the influence of insulin and LH independently and in combination on proliferation of adipocytes. Insulin and LH were added in a dose dependent manner (0, 10, 100ng/ml) to media (DMEM/adipocytes) in the absence of FBS following 24hr serum starvation of primary cultures. Controls included wells containing media only. All results were expressed relative to the standard curve in relation to cell number as seen in **Figure 26.**

3.5 Immunofluorescence.

Immunofluorescence (IF) uses primary antibodies to identify proteins of interest whilst secondary antibodies conjugated to a fluorochrome act by showing fluorescence. Excitation of fluorophores bound to the secondary antibody results in fluorescence at specific wavelengths and can be viewed using a fluorescent microscope. This technique therefore allows the localisation of target proteins within the cell. Fixation of the desired cells is required. This allows preservation of a sample in its native state. It is necessary to prevent diffusion or osmotic damage through expansion or shrinkage of cells. Permeabilisation of the cell facilitates access of the chosen antibody. Various methods of fixation exist including acetone or methanol, or a combination acetone:methanol (1:1). These two methods fall into the organic solvents category, which removes lipids and dehydrates the cell whilst precipitating the proteins [218]. Alternately cross-linking methods including paraformaldehyde preserve the cell structure by forming hydroxyl-methylene bridges between reactive end groups of adjacent proteins [218]. This method however can remove antigenicity. Determining the correct fixative immobilizes antigens and maintains the cell structure to as close to its native state as possible with minimal denaturation of the protein of interest.

It is necessary to inhibit non-specific antibody binding through a blocking procedure. The second phase of the protocol is primary antibody incubation of the cells, Following this, a complementary secondary antibody is added (anti-goat corresponds antibodies raised in goat etc.) and an external energy source such as a light of specific wavelength, excites the electrons and causes fluorescence which can be observed through a fluorescence microscope see **Figure 27.**

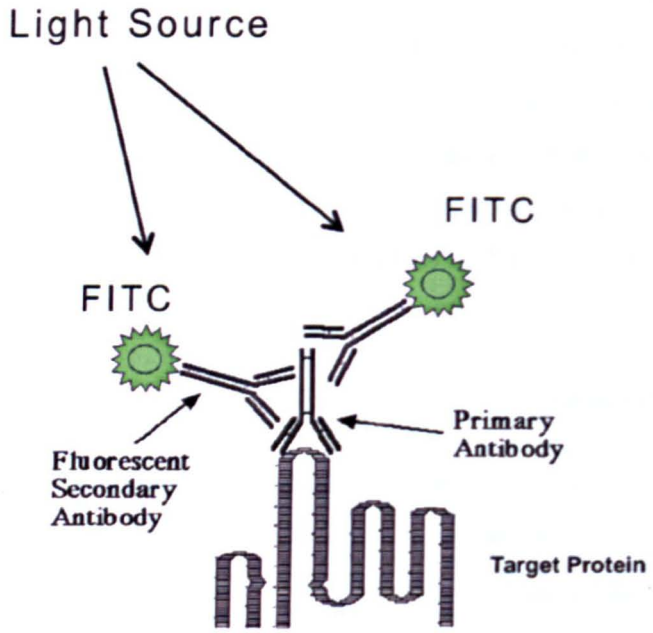

Figure 27. Immunofluorescence mechanics. Target proteins are located by the corresponding primary antibodies. The cell is then incubated with a secondary FITC conjugated antibody. The addition of a light source excites fluorophores and fluorescence occurs through energy release, which can be viewed via a fluorescent microscope revealing the location of the protein.

3.5.1 Immunofluorescence protocol: Fixation

All primary cells were probed using immunofluorescence in order to confirm their identity. Specific antibody markers were chosen and appropriate controls used to verify the protocol used (see **Table 3**). Leucocyte cultures were purified from blood and used as a negative control against all our primary cells (**Figure 28**). Leucocyte cultures were also probed using β-actin antibody to ensure a successful IF protocol.

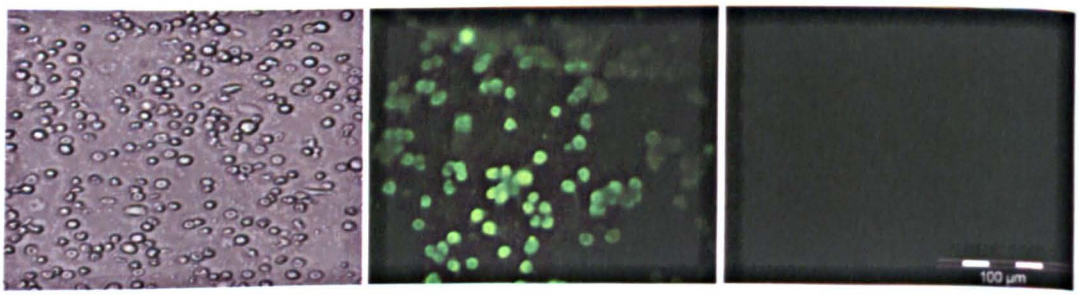

Figure 28. Human blood Leucocyte cultures grown to act as a negative control for antibody markers found in adipocyte and theca cells. Fluorescence shows β-actin antibody for positive control of IF protocol Blank micrograph on the extreme right, shows no fluorescence when cells were incubated in non-immuno serum in place of primary antibody. All images x 10 magnifications.

Cell Type	Primary Antibody	Control Antibody	Control Cells
Human Preadipocyte	anti-OB CYP17 LHR	β-actin	Leucocyte
Human Mature adipocyte	anti-OB CYP17	β-actin	Human mature adipocyte cell line Leucocyte
Human Theca	CYP17 LHR	LHR β-actin	CHO Leucocyte

Table 3. Table showing the primary antibodies used in this study to characterise the specific cell type and the supporting control antibodies and cells.

Due to the differences in fragility between theca and mature adipocytes both acetone:methanol and paraformaldehyde protocols were used to fixed cell cultures. However we found that the acetone:methanol protocol proved more successful as it led to less damage and maintained cell integrity.

Preadipocyte and theca cultures were grown to around 50% confluence at a passage between 2-3. In doing so enough space existed between the cells to allow identification of the cell morphology (*see 4.4.2.2*). It was however necessary to grow the mature adipocytes to confluence due to the protocol required in differentiation (*see 3.2.2*).

Cells were seeded at $5x10^3$ in 24 well plates and grown to required confluence (1-2 days) before careful removal of media and washing in ice cold PBS (x2). 300µl chilled acetone:methanol (1:1) (v/v) was added to each well and left for 10 mins before careful removal and further washes in ice cold PBS (x2).

3.5.1.1 Blocking of non-specific antibody binding sites

Fixed cells were blocked using 20% goat serum in PBS and left for 30 mins at RT. Following incubation, blocking solution was removed and the antibody of interest

diluted in PBS as required and added to the wells (*see* **Table 3**) (see appendix II for details). Cells were incubated overnight at 4°C with gentle rocking. All procedures were done alongside negative and positive control cells utilizing the same protocol. Further negative controls were prepared using fixed cells omitting the primary antibody and stored in PBS overnight at 4°C. Primary antibodies were carefully removed and all cells washed for 5 mins in ice cold PBS (x4).

3.5.1.2 FITC antibody and examination

A single isomer fluorescin isothiocyanate (FITC) tag was used bound to the chosen secondary antibody. This derivative of fluorescein emits green fluorescence on excitation at a wavelength of 494nm. Following PBS washes, secondary antibody at dilutions as per appendix II, was added to all cells and incubated at room temperature for 1 hour 30mins, wrapped in foil to prevent direct light bleaching or fluorophore excitation. The foil wrap was then carefully removed and all cells washed carefully in ice cold PBS for 5 mins (x7). Cells in PBS were then viewed in a dark room using Carl Zeiss Axiovert 25 Scope with Cell^F imaging software (Build 1131). Images were viewed and edited in Ziess LSM image browser V4.2.0.121.

3.6 Western blotting

Western blotting (immunoblot) is a widely used semi quantitative method for the detection of specific proteins from a given sample. It utilizes gel electrophoresis separating denatured proteins by weight/size. Proteins are then transferred to a nitrocellulose membrane and probed with antibodies specific to the target protein (*see 3.6* for detail). This study-utilised protein from lysates prepared from both tissue and cultured cells, allowing a comparison representative of physiological conditions. As a control, CHO cell cultures were grown and lysates created for use alongside all experiments. Original stocks of all controls were utilized through frozen stores at -80°C.

It is necessary to prepare lysates using various protocols (*see 3.5.1*) before western blotting.

3.6.1 Tissue homogenization

Tissue was collected from the Department of Obstetrics and Gynaecology at the Royal Derby Hospital (*see 3.1*) and immediately processed within the clinical sciences lab to limit protein degradation. Human follicles were finely chopped in homogenization buffer consisting of sucrose 300mM, tris base pH 7.4 25mM (Invitrogen Life Technologies), monothyioglycerol 10mM, EDTA 1mM, protease inhibitor cocktail 0.5% (v/v), 2.5% (v/v) igepal (see appendix). The tissue (0.5-1g) was then added to 3ml of homogenization buffer on ice and minced using a homogenizer (Hiedolph Diax 900) within a fume cabinet. The homogenate was then centrifuged for 30min at 250 x g at 4°C. The supernatant was removed, and placed in 2 eppendorf tubes. The remaining pellet contained homogenised tissue and was re-suspended in 200µl of solubilisation buffer consisting of Tris base pH 7.5 20mM, EDTA 10mM, sodium chloride 120mM, potassium chloride 50nM, dithiothreitol 2mM, protease inhibitor cocktail 0.5% (v/v) phosphatase inhibitor cocktail II 2.5% (v/v) igepal. This was then centrifuged for 10min at 250 x g 4°C allowing the detergent within the solubilisation buffer to solubilise cell membranes, releasing proteins and leaving sedimentation of the insoluble components. The supernatant was then stored at -80°. The 2-eppendorf tubes containing supernatants from the original step were then centrifuged for 1 hour at 250 x g at 4°C. The freshly solubilised supernatant from the 2 eppendorf tubes was then removed and stored at -80° or immediately used in protein estimation (*see 3.6.3*). The same protocol was used for adipocyte tissue however homogenization/ solubilisation buffer varied (see appendix).

3.6.2 Cell lysis

Cell cultures used in western blotting were grown to as low a passage as possible allowing for sufficient yield (2-4). Following treatment cell were lysed. Cells were carefully washed in HBSS (x2) and lysis buffer consisting of Tris base pH 7.5 25mM, sucrose 300nM, monothyioglycerol 10mM, EDTA 1mM, 1% (v/v) igepal, protease inhibitor cocktail 100µl in 100mls, phosphatse inhibitor II (100µl in 100mls). Following 5 mins incubation at room temperature cells were aspirated from the well

using a syringe and placed in eppendorfs and centrifuged to remove debris. These were either used for immediate protein estimation or stored at -80°.

3.6.3 Bicinchronic acid assay (BCA) method for estimation of protein concentration

The BCA method of protein estimation uses the reduction of Cu^{2+} to Cu^{+} in the presence of protein, which produces a measurable colour change in the reagent. This is quantified by measuring absorbance at 562nm through spectrophotometry. By preparing protein standards, comparisons can be made and protein estimations taken for specific tissue. Standards were prepared by serial dilution of a 10mg/ml stock solution of bovine serum albumin (BSA) ((0.9%) NaCl + 10µl of BSA 10mg/ml) to generate BSA concentrations at 0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1mg/ml. The standards were loaded in duplicate in a 96 well plate along with the unknown samples, with 200µl of BCA working reagent (4% copper sulphate ($CuSO^4$) in BCA reagent). This was then added to each well and incubated for 10min at 32 °C with gentle agitation. The absorbance of the standard and unknown samples, were read at 562nm on a plate reader (Thermo Electron Corporation Multi scan Spectrum). This information was taken and a standard curve generated (see **Figure 29**). Using this protocol with processed tissue/cell lysates, results were collated and examined against linear regression to determine protein concentration estimation.

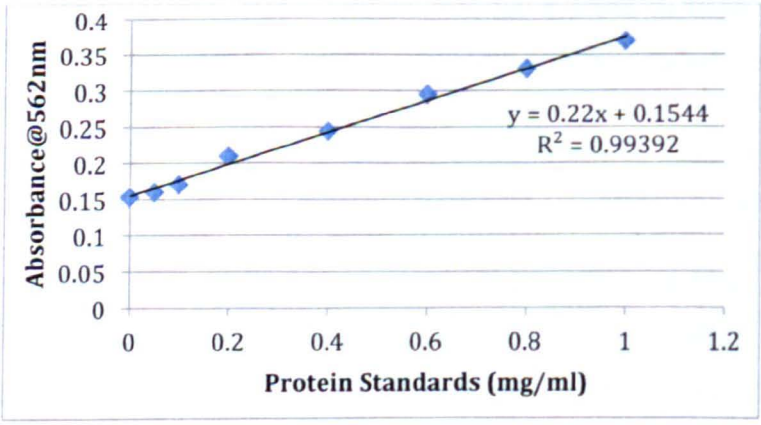

Figure 29. Graph showing the absorbance of known protein concentrations. Known protein concentrations were used to produce a standard curve. This could then be used to estimate unknown protein concentrations.

3.6.4 Preparation of gels (SDS-PAGE) and western blotting

Gels for western blotting were cast using glass gel plates. These were first cleaned using 70% ethanol and two plates secured using petroleum jelly within cast clamps. The acrylamide percentage was chosen in accordance with the molecular weight of the proteins being separated [26] (7,10,12%). High acrylamide percentage gels were used to separate low molecular weight proteins (10 – 15kDa). Low acrylamide percentages were used to separate high molecular weight proteins [26]. The resolving gel was prepared using 7-12% acrylamide stock (30%), resolving gel buffer consisting of dH_2O, Tris 1.5M pH 8.8, 0.1% (v/v) SDS, 0.001% (w/v) ammonium persulphate and 0.1% (v/v) Temed (see appendix). This was carefully syringed between the plates and left to set with a fine layer of butanol to prevent oxygen exposure (drying out and inhibition of polymerization) and promote polymerisation. The gel was left to set for ~30min. The stacking gel was prepared using 4% acrylamide stock (30%), stacking gel buffer consisted of ddH_2O, Tris 1M pH 6.8, 0.1% (v/v) SDS, 0.001% (w/v) ammonium persulphate and 0.1% (v/v) Temed. The butanol was removed with careful dH_2O washing and the stacking gel was added immediately followed by insertion of a lane comb and left to set for ~30min. Throughout this method The Bio-Rad mini-protein II electrophoresis unit was used (see appendix). The percentage gel chosen was based on the molecular weight of the proteins of interest with higher percentage gels allowing heavier proteins to be separated more easily. As these weights remained similar throughout a 10% gel was sufficient.

Protein concentration was adjusted to 10mg/ml using Laemmli buffer. The samples of interest were then denatured by exposing them to a hot plate set to 95°C for 5mins, before transferring to ice ready for electrophoresis. Each lane was loaded with 100µg of protein (see **Figure 31**). In addition, the first lane was loaded with 10µl of kaleidoscope pre-stained molecular weight markers to facilitate protein size determination (see **Figure 31**). The gels were then carefully placed into a bath of running buffer consisting of Tris (25mM), Glycine (192mM) and ddH_2O. This was run for 1 hour at 40mA (constant). The proteins were then electro-blotted onto nitrocellulose membranes in ice cold transfer buffer (tris base (24mM), glycine (80mM), 20% (v/v) methanol) within a BioRad miniprotean blotting system where a

sandwich of gel to nitrocellulose see **Figure 30** was added, and a charge of 100v (constant) run for ~ 2 hours at 4°C.

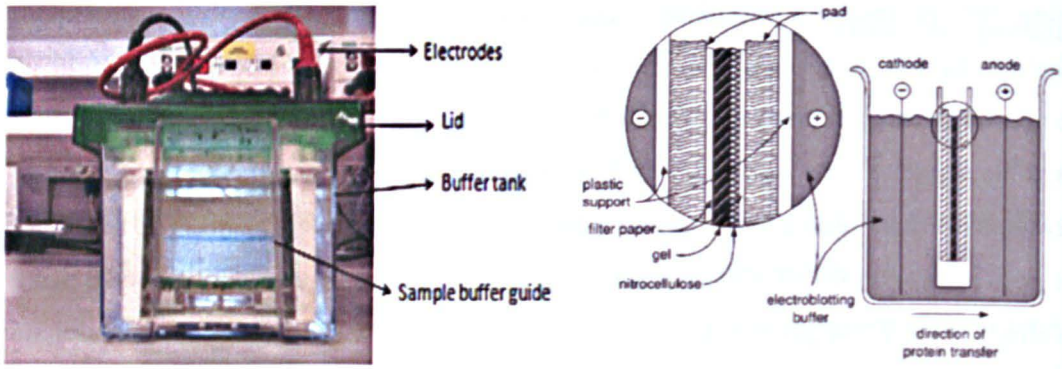

Figure 30. Bio-Rad mini-protein II electrophoresis unit. Each sample was carefully loaded into separate lanes. The blue dye front line shows the migration of the samples and the run was stopped when the dye front reached the bottom of the gel. Image obtained from http://www.currentprotocols.com/protocol/ns0519

Completed transfers were dismantled and the proteins stained in a bath of ponceau red (Sigma) for 10 min. These were then carefully rinsed within dH_2O and scanned to determine proteins had transferred efficiently (**Figure 31**). The membranes were then washed with distilled water to remove the ponceau.

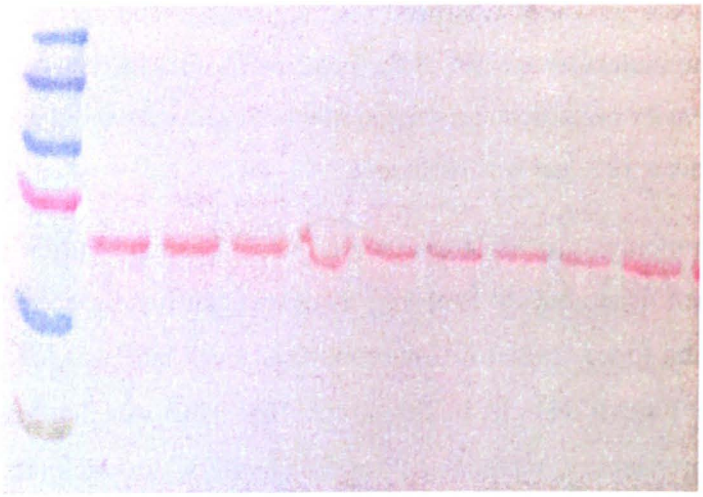

Figure 31. Ponceau Staining. Ponceau stain was used on the nitrocellulose following electro-blotting. The red staining shows the protein separation and transfer.

The nitrocellulose was then immersed in a bath of 5% Marvel milk and TBS (Tris (12.11g), NaCl (146.1g), dH_2O (5 l) for 1 hour, allowing blocking of the binding site regions on the nitrocellulose. This prevented any further interaction between the membrane and additional proteins i.e. the antibody. The relevant antibody was then

added at the correct dilution, in a 3% marvel solution (see appendix II) and incubated overnight with gentle agitation at 4°C. A washing procedure was then used with TBST (x6) for 10 minutes followed by TBS washes (x3) for a further 10 min each. The blots were then incubated with alkaline phosphatase (AP) conjugated secondary antibodies (for dilutions *see* appendix II)in 3% marvel for 2 hours at room temperature with agitation. The membrane was washed again as described above and incubated for 10min using enhancer and substrate solution (non-isotopic chemiluminescent detection system Immun-Star-Alkaline phosphotase kit (AP:Biorad)). This method utilises the enhancer and substrate that produces luminescence. The alkaline phosphatase (AP) method uses a hydrolase enzyme that removes phosphate groups to initiate a chemiluminescent reaction that can be viewed directly using a camera. Visualisation was enabled, by using 50µl of enhancer added to 2.5ml substrate evenly coating the nitrocellulose blot. The Chemi Doc (vers 4.2.1) imaging system was then used to visualise the immune-reactive bands on the membrane.

3.6.5 Positive controls

Western blotting is a long and complex procedure, which can take up to 48 hours to produce results. Multiple stages in the procedure therefore leave room for error. These may include inconsistent transfer, protein degradation and unequal protein lysate loading. We took necessary precautions where possible, including ponceau staining and positive/negative controls. CHO cell lysates were prepared, stored and used throughout all western blot procedures. When signs of degradation occurred these were replaced with lysates from the same passage of culture previously stored at -80°C. Negative lysates in the form of leucocyte taken from human blood (*see 3.5.1*) were also used.

Following each experiment for the protein of interest the same nitrocellulose was probed for β-actin, which was use as our loading control antibody due to its ubiquitous nature [219]. Reblot was used and the nitrocellulose incubated for 10 mins (ReBlot Plus, Chemicon International). This allowed previously bound antibody to be stripped away through an alkaline based solution. Blots were then washed in TBS-T (x3) for 10 mins and blocked using 5% marvel milk solution for 90 mins. The blots were then washed twice with TBS-T for 1min each before application of the β-actin

(1:8000 antibody dilution) in 3% w/v marvel milk/TBS overnight on a gentle shaker at 4°C.

3.6.6 Data analysis

All control blots were analysed alongside the results and exposure was kept constant to each protein of interest dependent on optimisation and resultant levels of over exposure. Once all areas of the blot had been normalized using a rolling disc option that allowed areas of background overexposure (dirty blot) to be considered. Both the average and total band intensities were measured. Variation against the β-actin was examined. The software also allowed calculation of the molecular weight of the proteins identified using the pre-stained molecular weight markers as a guide (see **Figure 31**). All western blotting experiments were repeated at least three times for each patient sample and both the mean ± SEM along with median ± interquartile range (IQR) were recorded (*see results*). Normality was tested using Shapiro-Wilk/Kruskal Wallis normality test within the prism software (vers. 5.0b). Multiple means for unpaired samples were compared using either one way analysis of variance (ANOVA) (parametric) with *Bonferroni post-hoc* test while paired observations were analysed using two tailed students' *t-test* (parametric). P-values <0.05 allowed for rejection of the null hypothesis.

3.7 Hormone measurements by ELISA

ELISA or enzyme linked immunosorbent assays allow the measurement of a specific protein within a solution. They rely on specific interaction between an epitope, and a matching antibody. Therefore the basis of all enzyme immunoassays (EIA) is the separation of specific or non-specific interactions through serial binding to a solid surface and quantification of the results via colour change examination. The coloured end product correlates to the amount of analyte present in the original sample. The process is rapid and allows examination of multiple samples in parallel. They were first developed independently and simultaneously by the research group of Peter Perlmann and Eva Engvall at Stockholm University in Sweden and by the research group of Anton Schuurs and Bauke van Weemen in The Netherlands as an alternate to radio-immunoassays in 1970 [220]. In some cases can allow for multiple analytes per well, highly sensitive readouts, and direct cell-based output.

Typically, 96 well plates are used and coated with either an antigen or antibody through adsorption or the passive attachment of a liquid to a solid surface to form a film. Once coated, blocking and detection steps are used to determine concentrations within the chosen sample (see **Figure 32**).

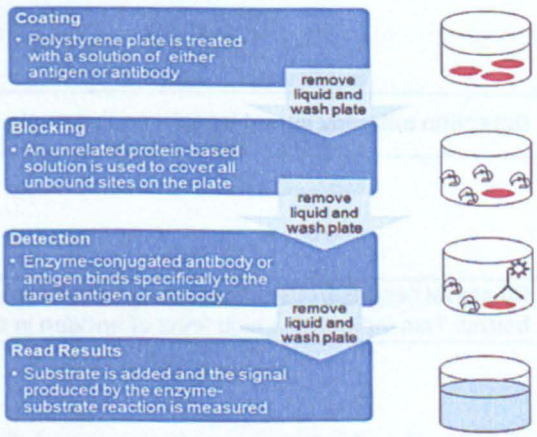

Figure 32. ELISA Technique. Protocol for basic ELISA showing the necessary stages including coating, blocking, detection and examination of results. Diagram taken from http://www.abdserotec.com/resources/elisa-technical-resources-and-troubleshooting.html.

Assays rely on surface binding for separation. Several washes are required between each step to remove unbound materials allowing for specificity. It is therefore

important that excess liquid is removed preventing dilution of the solutions added in later stages.

Four ELISA techniques exist allowing for a basis of assay formation. Direct, indirect, sandwich and competition. Our protocol relied on competition ELISAs for all our hormones of interest. The ELISAs were bought as ready-made kits that had been optimized to the specific hormones we were investigating allowing for reliable results. Competition ELISAs measures antigens/antibodies by examining interference within a know output i.e. using ready-made standard samples. An example of a competition ELISA can be seen in **Figure 33**.

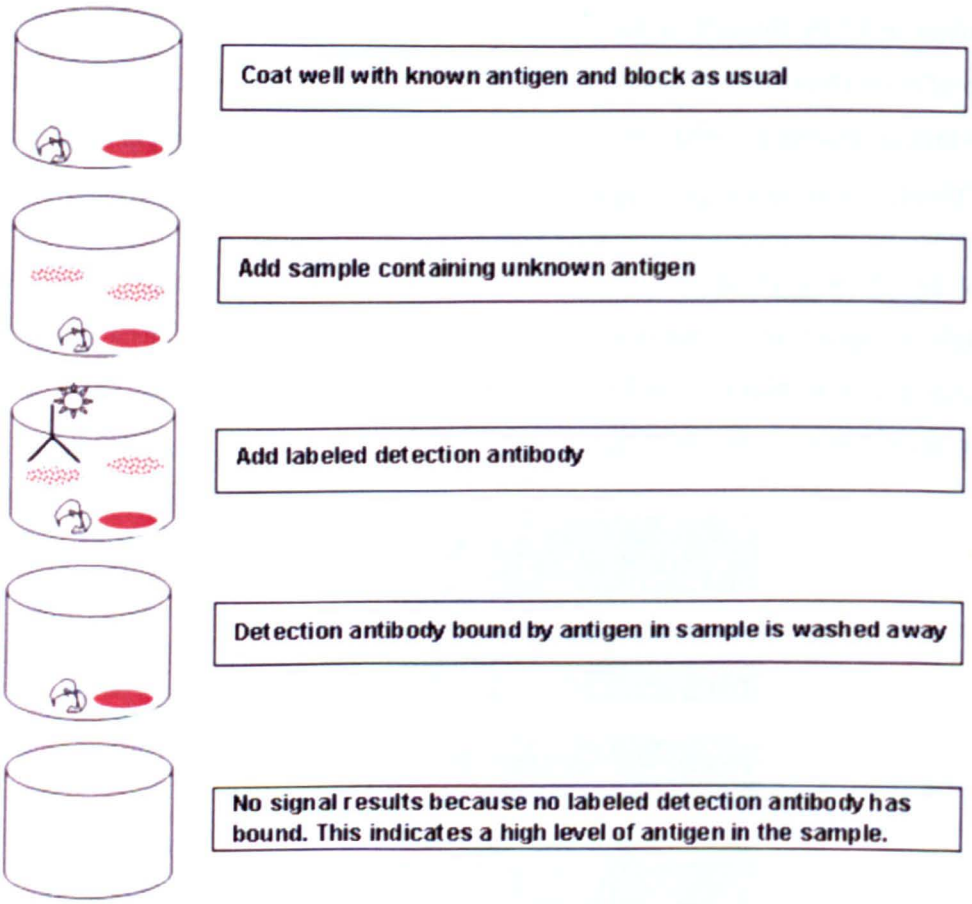

Figure 33. Competition ELISA. Bound and free antigen 'competes' for a labelled detection antibody. Image adapted from http://www.abdserotec.com/resources/elisa-technical-resources-and-troubleshooting.

An ELISA plate is prepared with a bound known antigen. The unknown sample is added and if the antigen within the sample is the same as the bound antigen then once the labelled detection antibody is added it will compete between free and bound

antigen. High sample concentration will cause detection antibody binding, which will then be removed during the washing process leading to reduced signal changes. A lack of similar antigen within the sample will lead to detection antibody binding to the bound antigen resulting in increased signal change. Therefore a reduction in signal intensity shows the existence of the antigen of interest within the sample. Signal changes were detected through colour changes measured via spectrophotometry at a wavelength of 450 ±10 nm.

3.7.1 ELISA standards

Three ELISA kits were chosen measuring progesterone, androstenedione and testosterone (results not shown). The progesterone ELISA (Ridgeway) allowed for measurement of samples to a sensitivity of 0.025ng/ml-0.39pmol/1000 cells using a standards range of known progesterone samples as supplied. Absorbance was measured (570nm) to create a standard curve (see **Figure 34**). The standard curves produced failed to form a linear result across the entire ranges of known samples however all results fell within an acceptable range for reliability. If higher values had been found within our unknown samples dilutions could be used to create a more reliable standards range. All results for the specific ELISA were obtained using this standard curve showing values falling within the ELISA range of sensitivity. Values were converted throughout to pmol/1000 cells; this allowed direct comparisons to published findings ([221, 222]). This would also allow for comparison of levels of metabolism in relation to the specific cell type.

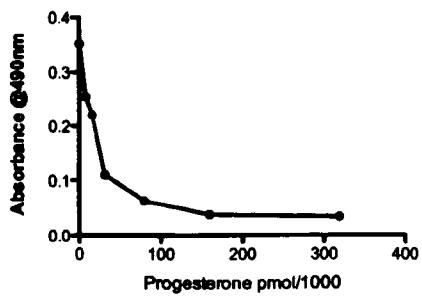

Figure 34. Standard curve showing ELISA progesterone levels through measured absorbance. Known levels of progesterone were measured using ELISA technique and levels of absorbance @490 taken to determine a standard curve for which all results could be measured by.

The androstenedione ELISA (GenWay Science) allowed for a sensitivity of 0.019ng/ml-0.03pmol/1000 cells) and standards of known androstenedione (as supplied) allowed to produce a standard curve to which all results were compared and produced (*see* **Figure 35**)

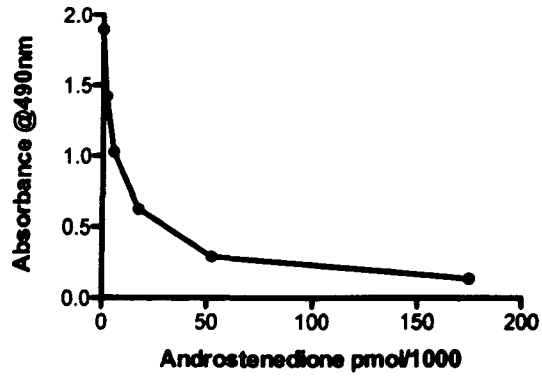

Figure 35. Standard curve showing ELISA androstenedione levels through measured absorbance. Known levels of androstenedione were measured using ELISA technique and levels of absorbance @490 taken to determine a standard curve.

3.7.2 Controls

Controls were used throughout all ELISAs and included supernatant from theca incubated with 100ng/ml insulin following three days treatment (*see 3.3*)(Intra/positive marker for both progesterone and androstenedione presence); osteoclast culture media following three days culture in standard media (DMEM) was used as a negative control for both progesterone and androstenedione levels (negative control). Media samples alone were also used as intra assay throughout, DMEM alone for adipocyte culture examination and DMEM/F12 alone for theca cell examination.

3.7.3 ELISA procedure

The procedure remained the same for all three ELISAs used. All ELISA plates were brought to room temperature and 10µl of standards/samples and controls added to duplicate wells. These included a blank well for comparison and removal of any background interference. 200µl of either progesterone/androstenedione/testosterone-enzyme label was added and left for 2.5 hours at room temperature covered and

gently shaken. The enzyme label was then removed and each well washed (x3) in 200µl of wash buffer for 2 mins at a time. Wash buffer was then removed and the wells tapped dry onto an absorbent tissue and air-dried. 200µl of alkaline phosphatase substrate was added to each well and incubated at room temperature for 30 mins. Colour development was immediately measured using a spectrophotometer (progesterone 570nm, androstenedione/testosterone 450nm OD) and results analysed by comparison to standards (*see* **Figure 34, Figure 35**).

3.7.4 Data analysis

All control samples were analysed alongside the results and OD examination was kept constant to the ELISA used (progesterone 570nm, androstenedione/testosterone 450nm OD). All samples were analysed as duplicates. Furthermore an intra assay sample of insulin treated and untreated 3 day media was used from frozen stock throughout all experiments and media blanks included dependent on the cells used (DMEM/high glucose and DMEM/F12). Once known, sample standards were analysed (taken from each ELISA kit) standard curves were created by collating spectrophotometry data within Microsoft Excel 2010. This data was organized and transported to the prism software (vers. 5.0b) where once interference was removed from each result, was interpolated against the standard curves to give hormone secretion data (ng/ml). This was then converted to pmol/1000cells for standardization and normalized using Shapiro-Wilk/Kruskal Wallis normality test. Multiple means for unpaired samples were compared using either one way Analysis of variance (ANOVA) or un-paired *t-test* (non-parametric) with *Bonferroni post-hoc* test while paired observations were analysed using two tailed students' *t-test* (parametric). P-values <0.05 allowed for rejection of the null hypothesis.

4.0 CULTURE AND CHARACTERISTICS OF 3T3-L1 CELLS AND PRIMARY ADIPOCYTES FROM NON-PCOS AND PCOS WOMEN

4.1 Introduction

Mature adipocytes show little proliferative ability and controversy exists as to whether they have this capability [223]. *In vitro* differentiation is therefore a suitable alternative. This allows establishment of high cell yields through preadipocyte cultures and maturation to adipocytes through differentiation treatments.

It is only within the last 20 years that adipose tissue has been considered to act as an endocrine organ [207, 224] following the identification and characterization of leptin discovered by Zhang *et al.*, 1994 [225]. Research has since determined various bioactive peptides (adipokines) that allow systemic signalling to adipocytes (**Table 4**). It has also been shown that adipocytes express various receptors that mediate adipocyte responses to many traditional hormones including insulin, androgens and eostrogens [226-228]. With the importance of obesity and the association this has with metabolic abnormalities such as insulin resistance (diabetes) and hyperinsulinemia, and conditions including cardiovascular disease, and metabolic syndrome, examination of adipocyte function and subsequent malfunction is of great clinical importance.

Adipocyte-derived proteins	Endocrine function
Leptin,	Hormone energy regulator.
TNF-α, IL-6	Cytokines and cytokine- related proteins. Immune system intercellular mediators.
Plasminogen activator inhibitor-1 (PAI1), Tissue factor	Proteins involved in the fibrinolytic system
Adipsin (complement factor D), Complement factor B, ASP, Adiponectin	Complement and complement-related proteins that allow antibody binding and immune reaction.
Lipoprotein lipase (LPL), Cholesterol ester transfer protein (CETP), Apolipoprotein E NEFAs	Lipids and proteins for lipid metabolism or transport and use in steroidogenesis
Cytochrome P450-dependent aromatase, 17β-HSD, 11HSD1	Enzymes involved in steroid metabolism

Table 4. Examples of adipocyte derived proteins and their endocrine function. Many bioactive peptides known as adipokines have been determined since the discovery of endocrine function of adipose tissue.

Although PCOS has always been considered to be a reproductive disorder, its metabolic implications are often highlighted, with insulin playing a significant role in its pathogenesis [159]. Approximately 50–70% of women with PCOS have insulin resistance and subsequent hyperinsulinemia [229]. This has major implications for metabolic abnormalities and hyperinsulinemia has been associated with increased ovarian androgen synthesis with Nestler (1989) showing that hyperinsulinemia in obese PCOS women may directly increase serum testosterone [230, 231]. Although this is believed to be a major contributory factor toward hyperandrogenic PCOS, it is not seen as solely responsible. Research into insulin actions on metabolic function within target tissue in comparison to the ovary is therefore of clinical relevance with regard to PCOS.

With 50% of PCOS women suffering from obesity, associations to PCOS and adipocyte dysfunction have been made [115, 232, 233]. One overriding factor seems to be abdominal fat distribution regardless of BMI [234]. In whole adipose tissue

samples taken from central abdominal deposits, data suggest that subcutaneous (SC) adipose tissue has higher rates of both androgen synthesis from precursors and androgen inactivation than omental fat (OM) [191]. However comparisons in levels of androgen secretion between PCOS and non-PCOS have yet to be carried out.

Research into abnormalities within PCOS adipose tissue is a fairly new area of study possibly due to the relatively recent acceptance of adipose as an endocrine organ and the association this may have with PCOS as an endocrinological disorder. Comparative studies into SC adipocytes from women with PCOS have revealed morphological variations with a 25% increase in adipocyte cell size seen in PCOS samples [235]. Other studies have shown a decrease in hormone-sensitive lipase expression and adiponectin levels in PCOS women which maybe suggestive of further biochemical abnormalities [232]. Further to this, Dickerson *et al.*, (2010) have shown that testosterone treatment of SC PCOS adipocytes was linked to reductions in adipokines and may point to PCOS adipocyte impairment being secondary to hyperinsulinemia [236]. This study therefore suggests possible variations between non-PCOS and PCOS adipocytes may exist in the pathogenesis of PCOS warranting further investigation.

In the present study, we sought to compare adipocytes from PCOS and non-PCOS women in order to determine any dysfunction (with particular focus on androgens), that may exist and obtain a more detailed insight into adipocyte function. Specifically, the focus of this Chapter includes an examination of adipocyte proliferation and differentiation as well as expression of adipokine receptors and hormonal influences on metabolism.

We also sought to determine the effects of LH and insulin on SC hormonal secretion to determine any variation within or between non-PCOS and PCOS SC adipose samples. LH and insulin were selected based on their well-characterised association to PCOS.

In order to investigate hormonal influences on adipocyte metabolism in women with PCOS, it was necessary to establish a workable human primary adipocyte cell culture model. Initial protocol optimization of culturing and differentiating adipocytes was achieved using the 3T3-L1 mouse cell line [237]. To date, this fibroblast cell line has

been widely used as a reliable model in the examination of the influence of insulin in adipocyte signalling pathways and pre and mature adipocyte intracrinology. This has led to discoveries such as insulin regulation of leptin secretion and TC10 modulation of insulin-stimulated GLUT4 translocation [238-241]. Collison *et al.*, (2000) were the first to examine the direct effect of sex steroids on peripheral insulin-sensitive tissue utilizing the 3T3-L1 cell model, supporting the role of oestrogen's influence in reduced insulin stimulated glucose uptake [242, 243].

The 3T3-L1 cell line has also been used as a model in which the proliferative effects of LH releasing hormone agonist (LHRH) and its involvement in prostatic cancer treatment were shown to reduce tumour growth [244]. It was concluded that the mechanism involved may be the suppression of the pituitary-testicular axis and subsequent reduction in testosterone secretion [242, 244].

The availability and supporting background studies have made the 3T3-L1 cell line a suitable animal cell line for our investigations but only with regard to determining suitable culture and differentiation protocol. No studies have utilised this model in respect to PCOS dysfunction and biochemical alterations from immortalisation gave limitations to adipocyte metabolism results. Therefore all comparisons were limited to our primary human model. Whilst the 3T3-L1 preadipocyte cultures required straightforward maintenance, establishing maturation of these cultures was more complex [237]. Although protocols exist for culturing primary mature adipocytes through ceiling methods, cell lysis has been shown to occur within 72 hours due principally to a lack of access to available nutrients within the media as a result of the lipid buoyancy of the cells [245]. In addition, primary mature adipocyte cultures have yet to be validated in their ability to retain all of their *in vivo* function [246], although it is unlikely that culture models mimic exactly *in vivo* functions.

4.2 Objectives

i. To successfully culture a viable 3T3-L1 cell line and differentiate to mature adipocytes for later use in expression studies with a view to transfer protocols to a human model.

ii. Successfully establish primary human preadipocyte cultures from abdominal SC adipose biopsies and achieve differentiation of confluent preadipocyte cultures.

iii. To compare proliferation and differentiation in adipocytes cultured from adipose tissue of normal women and those with PCOS.

4.3 Method

Cultures of the 3T3-L1 cell line at a passage of 20 were taken from liquid nitrogen storage and grown to confluence (*see 3.2.1*). Once confluent, normally within 7 days, preadipocytes were transferred to T75 flasks to increase yield. On achieving 80% confluence, preadipocytes were then plated in 24 well plates at a density of $2x10^5$ per well and grown to 70-80% confluence for differentiation within an incubator at 5% CO_2/air at 37°C.

Primary preadipocyte culture: In order to establish primary human preadipocyte cultures, SC abdominal biopsies were processed using enzymatic digestion (*see 3.2*) and the remainder filtered through 200μm nylon cell microsieves (BioDesign Inc., N200c)(*see 3.2.2*) This method was a variation of a technique used previously in our lab (Wood *et al.;* 2005) and originally established by Quinkler *et al.,* (2004*)* It was necessary to carefully separate all adipose tissue from the sample prior to digestion removing all vascular and collagen components, minimising contamination from other cell types [247, 248].

Primary cultures were prepared from SC biopsies and grown to around 80% confluence before differentiation over the course of 3-5 weeks as described in Chapter 3. Maturation of adipocytes was determined using oil red O staining of the lipid droplets (*see 3.2.2.1)* [237].

Human adipocyte proliferation was assessed using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay allowing

colorimetric assessment of cell proliferation and viability. Adipocytes were cultured over 5 days and proliferation assessed daily using the chemical reduction of MTS to formazen (*see 3.4*). Absorbance measurements were made by spectrophotometry at a wavelength of 490nm.

4.4 Results

4.4.1 Culture and differentiation of 3T3-L1 preadipocytes:

Figure 36 shows 3T3-L1 preadipocytes grown to 90% confluence over 7 days. The cells were arranged in clusters and became tightly packed producing a cobblestone appearance allowing for stabilisation and support of the cells. For this reason the initial differentiation media (*see 3.2*) was added to cultures at around 70-90% confluence. In doing so, all preadipocytes underwent growth arrest. This also occurred with increases in cell number so may be a result of cell to cell contact, which is believed to initiate adipocyte conversion as well as leading to secretion and exposure to initiation signals (CCAAT/enhancer binding protein a (C/EBP-a) and PPAR-γ,) of neighbouring cells [249]. This, along with the solution required to initiate differentiation, allowed for simultaneous maturation of the preadipocytes to mature adipocytes and prevented only partial maturation of cell patches within fully confluent cultures. Although preadipocytes adhered tightly to the plastic substrate, at this stage we found it necessary to grow the cells to confluence thus creating a sturdier matrix before differentiation. **Figure 37** shows 3T3-L1 cells following 4 weeks differentiation treatment. Following initiation of differentiation (*see 3.2.2.1*), the cells begin to elongate weakening the support matrix of their neighbouring cells and could be seen as cells breaking away from the cultures to form patchy grouping, gaps between or individual cells. Occasionally this action would lead to cells detaching from the base of the plate and floating within the media. Small lipid droplets formed in approximately 70% of the cells. Using light microscopy, these droplets were seen as light circular spots within the cells and can appear blurred due to the need to focus on a higher plane within the z-axis than would be needed for flat, adherent cells. The buoyancy of the lipids within the cells caused the cells to detach and required careful handling during media changes. Lipid droplet size increased from 10 ± 7 μm ($n = 10$) to up to 100 ± 15 μm ($n = 10$) with maturation as seen in **Figure 38** and **Figure 39**. As the cells continued to alter in morphology, becoming rounder, the distance between neighbouring cells continued to increase. This coupled with increased lipid buoyancy led to sheets of cells tearing away from the base of the well and loss of cultures. Daily monitoring was required towards the end of differentiation in order to minimise such losses.

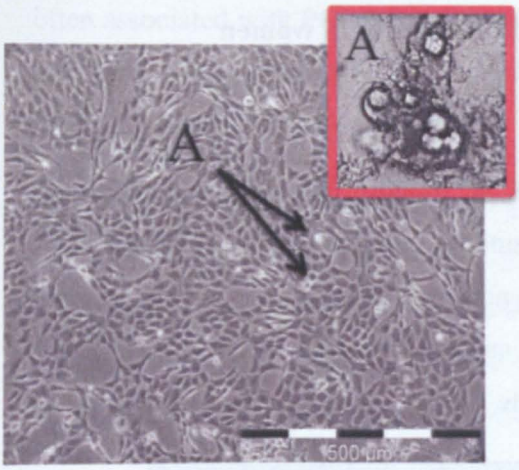

Figure 36. 3T3-L1 Preadipocytes 7 days following culture. Preadipocytes maintained at around 90% confluence. Preadipocytes are seen as flat and beginning to form a tight mosaic formation. (A) Shows preadipocytes beginning to differentiate.

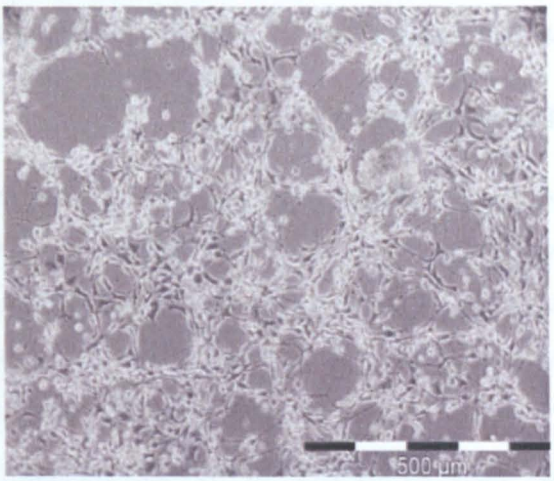

Figure 37. 3T3-L1 Adipocytes. Preadipocytes following 4 weeks of routine media changes occurring every 3 days with media consisting of insulin, IBMX and dexamethasone. Approximately 70% of the preadipocytes have undergone differentiation showing developing lipid droplets.

Validation of adipocyte differentiation was confirmed using, Oil Red O staining. Oil Red O is fat-soluble and stains neutral triglycerides red [250]. **Figure 39** shows both large lipid vacuoles and smaller lipid accumulation with red staining by Oil Red O.

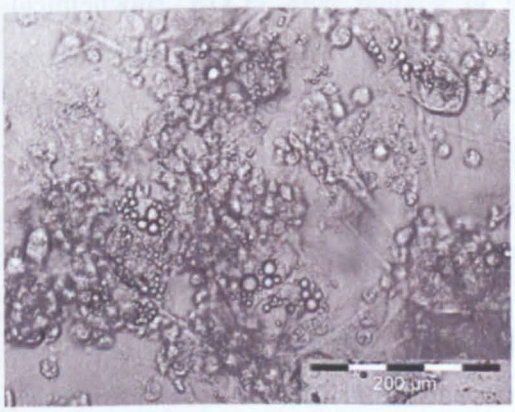

Figure 38. 3T3-L1 Adipocytes. Preadipocytes after 6 weeks in vitro. Preadipocytes have undergone differentiation into mature adipocytes as seen by the extensive accumulation of lipid droplets.

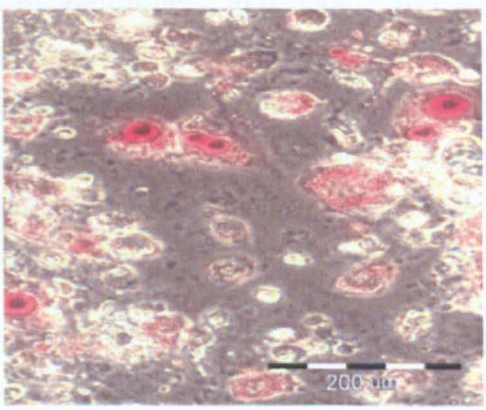

Figure 39. 3T3-L1 Adipocytes. Preadipocytes following 6 weeks differentiation stained with Oil red O. Red staining shows lipid droplets and subsequent maturation of adipocyte.

4.4.2 Human adipocyte cultures from non-PCOS and PCOS women

Patient demographics

Adipose tissue biopsies (~5g) were taken from SC adipose tissue of the abdominal wall of participating women either during a planned surgical procedure or under local anaesthesia in the outpatient clinic (for women not undergoing surgery). Two groups were selected, normal women (control group) and women with PCOS. (*See 3.1 for selection criteria*). Data are summarised in **Table 2**

Parameter	Normal (n=17)	PCOS (n=10)	P
Age (yrs)	33.8 (24-44)	30.5 (25-38)	(NS)
Body Mass Index (kg/m^2)	26.4 (17.6-33.1)	32.3 (19.8-37.7)	*
Insulin (pmol/l)	26.0 ± 5.8	118.6 ± 31.8	***
Testosterone (nmol/l)	1.4 ± 0.2	2.35 ± 0.4	*
Luteinising Hormone (nmol/l)	6.7 ± 1.4	29.86 ± 11.2	**

Table 2. Patient demographic of cultured samples. Data summarised as mean (range) / SEM (±) * P<0.05 ** P <0.01 *** P<0.001.

The age range of our samples allowed cultures to be closely matched for comparisons of PCOS and non-PCOS women. **Table 2** shows that the BMI between our age matched groups was significantly increased in women with PCOS. Biochemical variations between the groups existed with significant increases in testosterone (>2.9nmol/l), insulin (>170 pmol/l) and LH levels symptomatic of PCOS women. Recent studies have shown that DHEAS is less reliable than testosterone with only 5% of hirsute patients examined showing elevated levels [213]. For this reason we measured free testosterone levels (*see 3.1*).

Significant increases were seen in BMI, insulin, testosterone and LH within the PCOS samples, however in some women (n= 6), free testosterone did not exceed 2.9 nmol/l, typically related to the condition. Studies within the hyperandrogenic hursuitism phenotype have shown that 72% of patients examined had normal free testosterone levels with 55% of these also showing normal FAI. These results also showed increased androstenedione levels [213]. PCOS diagnosis was also supported by ultrasound presence of polycystic ovaries within our sample group. A significant variation across groups with LH secretion of which 90% of PCOS sample (n= 9) had LH levels exceeding normal levels (follicular phase >13 nmol/l). LH hypersecretion is

often associated with PCOS and LH/FSH ratios are often used as a diagnostic marker, although not independently. A strong correlation between LH levels and PCOS was seen within our samples (see **Table 2**).

Although the PCOS group showed significant increases in insulin secretion against the non-PCOS, the insulin levels typically seen within PCOS in accordance with hyperinsulinemia was only found within around 50% of our PCOS sample group. This allowed us to split this sample group between non-hyperinsulinemic-PCOS / hyperinsulinemic –PCOS for further examination (*see* **Table 5**).

Parameter	Non hyperinsulinemic PCOS (n=4)	Hyperinsulinemic PCOS (n=6)	P
Age (yrs)	29.33 (25-35)	32.25 (29-38)	NS
Body Mass Index (kg/m^2)	28.33 (19.8-35.4)	30.28 (22.6-37.6)	NS
Insulin (pmol/l)	48.44 ± 12.49	190 ± 32.32	0.002**
Testosterone (nmol/l)	1.50 ± 0.10	3.05 ± 0.62	0.032 *
Luteinising Hormone (nmol/l)	11.25 ± 3.3	26.75 ± 8.4	NS

Table 5. Patient demographic of PCOS cultured samples. PCOS samples with hyperinsulinemia against non-hyperinsulinemia. Data summarised as mean (range) / SEM (±) * P<0.05 ** P<0.01 *** P<0.001.

Examination of BMI between hyperinsulinaemic PCOS and non-hyperinsulinaemic PCOS showed no significance, suggesting no correlation exists between increased insulin and BMI, although our dataset is small. When examining our biochemical results, significant increases in testosterone levels found in our hyperinsulinemic PCOS women was seen.

4.4.2.1 Culture of non-PCOS and PCOS subcutaneous adipocytes

Of the 27 samples processed (n=7 non-PCOS / n=10 PCOS), 24 were successfully established in culture taking around 14 days to reach confluence in T25 flasks before careful trypsinisation to T75 flask in order to increase cell yield (**Figure 20**).

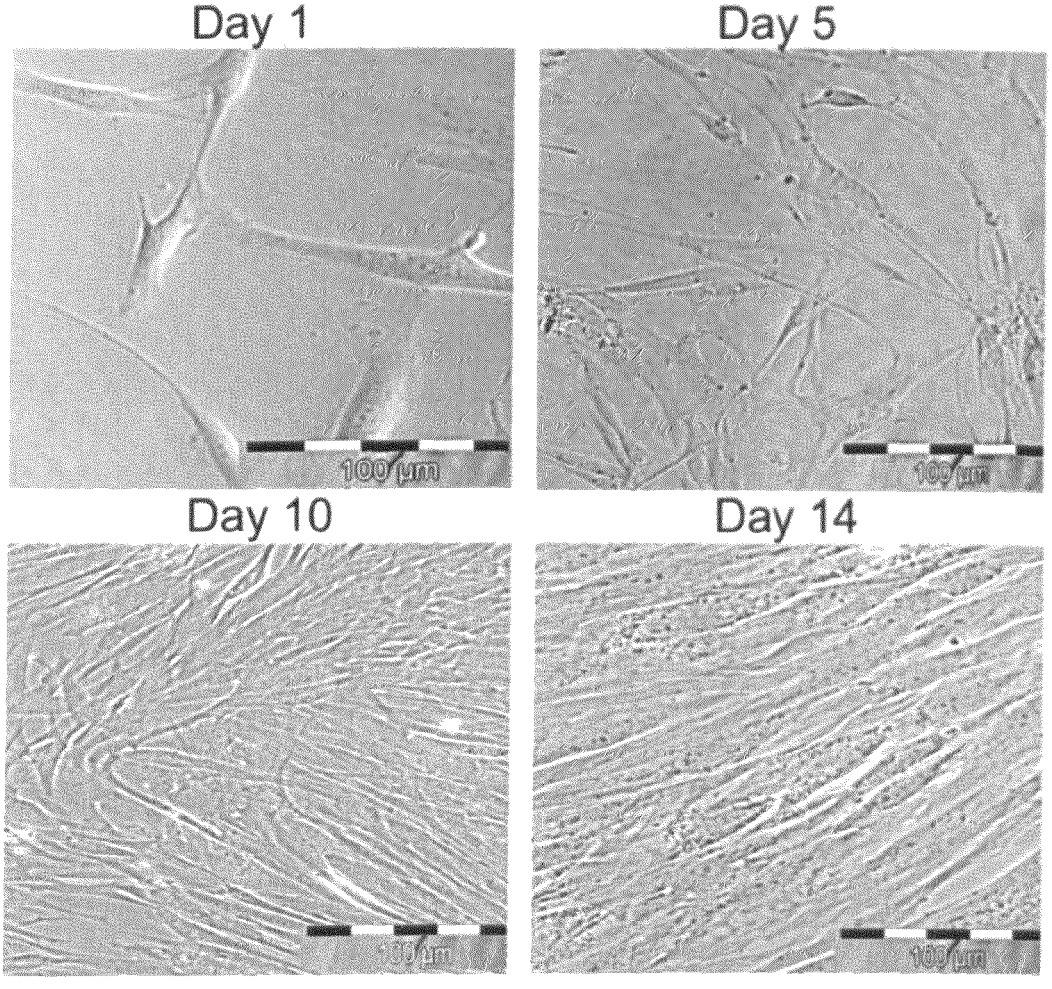

Figure 40. 14 Days *in vitro* Human Preadipocytes proliferation. Preadipocytes maintained in 10% serum DMEM. Morphologically the cells are elongated and flat. By day 10 the cells begin to form a tight matrix until Day 14 when 100% confluence gives a striated effect and firm adherence to the base of the flask.

Morphologically the cells are fine elongated and spindle shaped. Once grown to 100% confluence, they formed a tight cellular matrix that allowed strong adherence to the flask and as a result fibronectin coating was not required as suggested by Quinkler *et al.*, (2004)[247]. Similar to the 3T3-L1 model, it was necessary to regularly maintain the cells with daily checks and media changes every two days. This helped to prevent

sporadic differentiation. Examination of both 3T3-L1 and human primary preadipocyte cultures (n=6) showed that following 80-90% confluence, failure to add differentiation media caused sporadic differentiation of cell patches. If these cells then underwent the differentiation procedure via media treatment these patches would eventually de-differentiate with observations showing a noticeable decrease in lipid droplets throughout the cultures. Observations showed that these patches would then detach from the well plate leaving cellular debris typical of cell death. With the cellular matrix broken the remainder of cell would eventually detach halting maturation and cellular debris suggested signs of cell death.

Careful maintenance, suitable confluence and the addition of differentiation media immediately as the cells reached 80-90% confluence therefore allowed colony wide maturation of preadipocytes obtained through our protocol as recommended by Bujalska *et al.*, (1999)[251].

4.4.2.2 Characterisation of preadipocytes

SC biopsies required separation and processing to establish primary fibroblast cultures in order to minimise cellular contamination occurring during this process. Immunofluorescence and western blotting techniques were used to identify preadipocyte markers (anti-human OB R&D Systems (AF398)). All samples were run against positive (β-actin and commercially available human adipocyte cell lines) with leucocytes serving as a negative control against adipocyte markers (*see 3.5.1*).

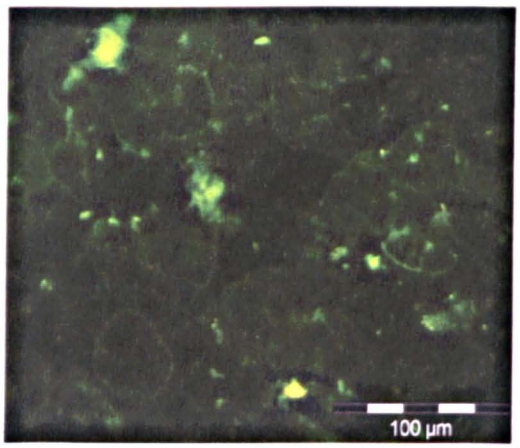

Figure 41. Immunofluorescence of mature human adipocytes. Mature adipocyte cells stained with anti-OB antibody and FITC conjugated secondary antibody. Anti-OB immunofluorescence is localised to the cell membranes.

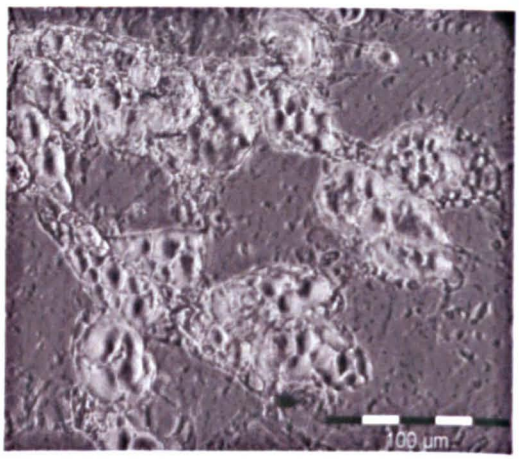

Figure 42. Bright field micrograph of mature human adipocyte 6 weeks *in vitro*. Mature human adipocytes differentiated to around 50%. Bright field allows determination of cell morphology and lipid droplets can be seen in the mature adipocytes.

Figure 42 and **Figure 41** show mature human adipocytes in bright field and fluorescent staining allowing cellular morphology to be seen clearly. **Figure 41** shows leptin was predominantly expressed within the cytoplasm surrounding the lipid droplets where it is known to localise to the ER [252]. Negative controls (leucocyte culture and incubating in secondary antibody alone) did not display staining for leptin but staining for β-actin was seen. Adipocytes from a commercial primary adipocyte cell line (PromoCell C-12730) and β-actin positive controls also supported findings with leptin seen staining human adipocyte cultures as well as β-actin positivity.

4.4.3.1.2 Western blotting

Expression of leptin protein was seen in both the human adipocyte cell line and primary adipocyte cultures through western blotting.

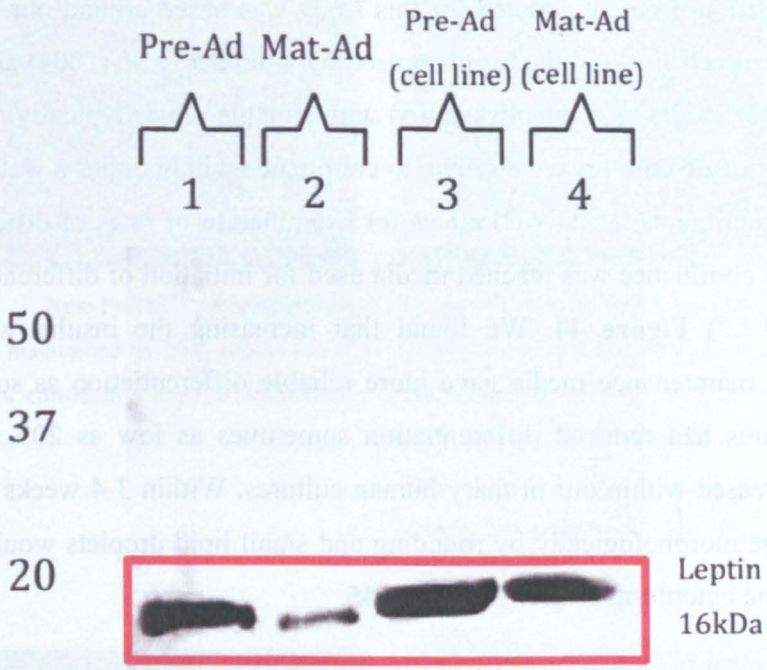

Figure 43. Western blot of leptin expression in preadipocyte (Pre-Ad/lane 1 and 3) and mature adipocyte (Mat-Ad/lane 2 and 4) lysates (n=2 vs. 2). Preadipocyte (lanes 1-3) and mature adipocyte lysates (lane 2-4) were probed using the polyclonal antiOB antibody. Exposure at 640sec shows band at predicted 16kDa, which were observed to be more abundant in preadipocyte samples. The blot was then stripped using reblot and re probed using β-Actin to control for protein loading l. β–Actin shows exposure density readings. Human mature adipocyte lysates were used as positive controls (lane 3-4).

Western blotting of primary human adipocyte lysates and human adipocyte cell line, from both preadipocyte and mature adipocyte cultures were used to examine and further support characterization of our primary culture. Anti-leptin (OB) antibody probe (anti-human OB R&D Systems (AF398)) was used as a positive marker in both the preadipocyte and mature adipocyte lysates of primary and cell line harvests (*see 3.6*). **Figure 43** shows positive band exposure at 640sec representative of leptin expression in all lysates (lane 1-4). Lane 2 primary mature adipocyte lysates shows

weaker exposure suggesting reduced leptin when compared to mature adipocyte lysates within the cell line (lane 4).

4.4.3 Comparison of differentiation in non-PCOS and PCOS adipocytes

The differentiation protocol adopted for this study was based around our experience with our animal cell line and the protocol used by Quinkler *et al.* (2004) and Chen *et al.*, (1997)[247, 253]. Once established and with suitable yield (typically passage 3), human preadipocyte cultures were grown to confluence within either 6 well plates for expression experiments, or 24 well plates for examination of rates of differentiation. Once 70-80% confluence was reached media used for initiation of differentiation was added (*see 3.2.2*) **Figure 44**. We found that increasing the insulin within both initiation and maintenance media gave more reliable differentiation as some of our original cultures had reduced differentiation sometimes as low as 20%. This was therefore increased within our primary human cultures. Within 3-4 weeks cells were seen to change morphologically by rounding and small lipid droplets would begin to form within the cytoplasm as seen in **Figure 45**.

We found that enlarged lipid accumulation did not exceed 70% of the differentiated culture, which took 6-9 weeks dependent on the culture (**Figure 47**). At this point cells would begin to differentiate and this would eventually lead to cell death.

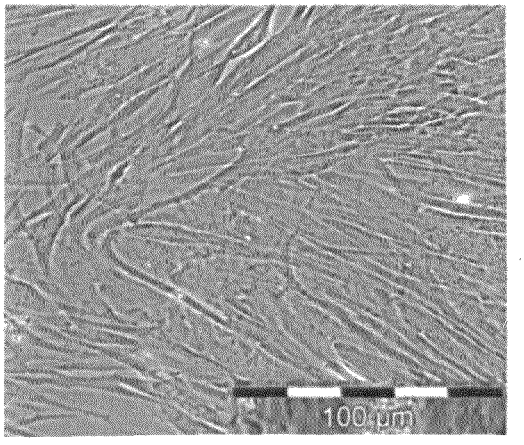

Figure 44. Non-PCOS Preadipocytes. Preadipocytes maintained in 10% serum DMEM at around 90% confluence prior to initiation of differentiation.

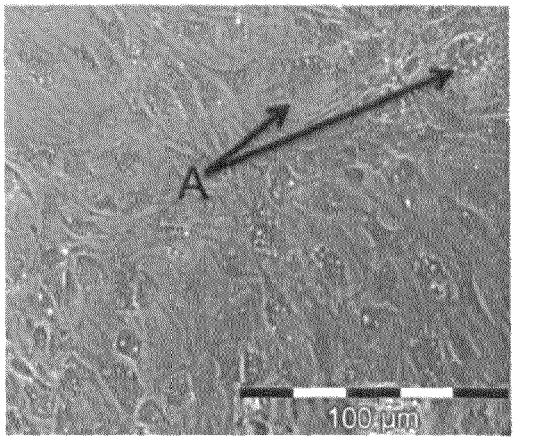

Figure 45. Non-PCOS Preadipocytes following initiation of differentiation and 3 weeks in maintenance media. Routine media changes every 3 days following initiation of differentiation. **(A)** shows the elongated preadipocytes beginning to round and acquire small lipid droplets.

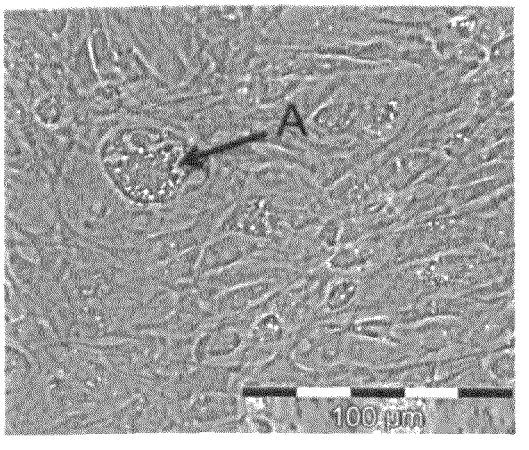

Figure 46. Non-PCOS 6 weeks differentiation. **(A)** Shows rounded adipocytes increasing in size and accumulating more small lipid droplets.

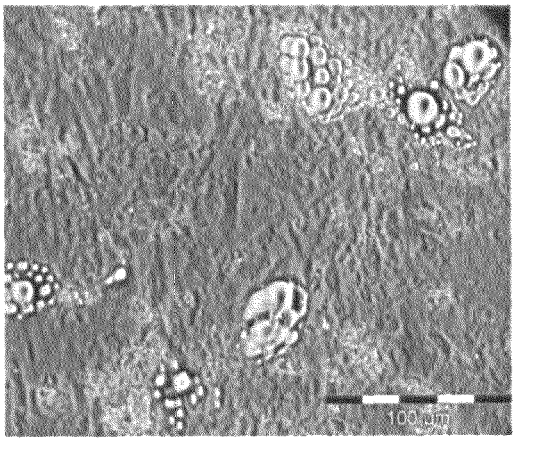

Figure 47. Non-PCOS 8-9 weeks differentiation. 70% of the cultures are have filled with lipid droplets which have increased in size.

Similar to the 3T3-L1 mouse cell line, Oil Red O was used to validate maturation of the adipocytes. **Figure 48** shows both large lipid vacuoles and smaller lipid accumulation with red staining by Oil Red O.

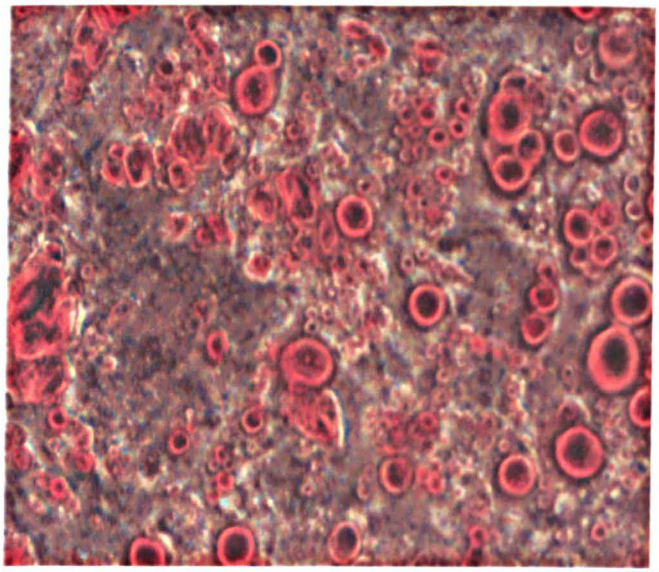

Figure 48. Non-PCOS 8-10 weeks differentiation Oil Red O Staining. 70% of the cultures have matured with large lipid droplet formation shown through Oil Red O staining. 10x magnification

By staining both non-PCOS and PCOS cultures with Oil Red O. comparisons in morphology could be made. Age and BMI matched cultures at $2^{nd}/3^{rd}$ passage were used to examine lipid droplet formation. Comparison of non-PCOS and PCOS lipid droplet formation in week 4 of differentiation were made. Although lipid accumulation was not quantitatively assessed, observations consistently suggested (**Figure 49**, **Figure 51**) samples from PCOS women had a greater number of mature cells based on the prevalence of larger lipid droplet formation. Measurement of the larger droplets within PCOS and non-PCOS mature adipocyte cultures following 10 week differentiation showed PCOS lipid droplet sizes at 80 ±29.3μm (n=10). Typically non-PCOS adipocytes, lipid droplets matured to 30 ±12.7μm (n=10) in size over a 10 week period.

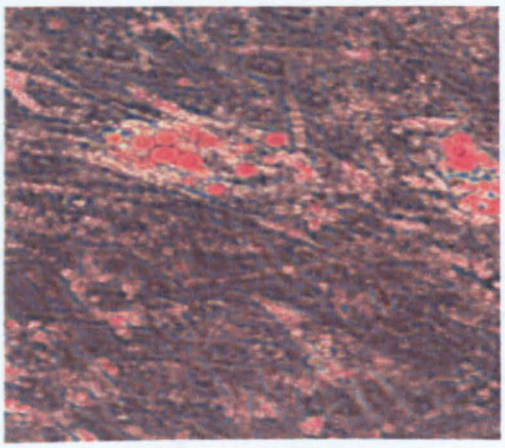

Figure 49. 4 weeks differentiation of non-PCOS adipocytes with Oil Red O Staining. 50% of the cultures have filled with small lipid droplet. Larger droplets are beginning to form shown through Oil Red O staining. 10x magnification.

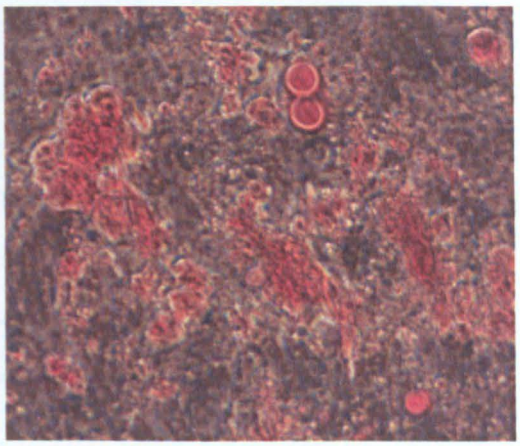

Figure 50. 9 weeks differentiation of non-PCOS with Oil Red O Staining. 80% of the cultures have filled with lipid droplet. Larger droplets show fully matured cells through Oil Red O staining. 10x magnification.

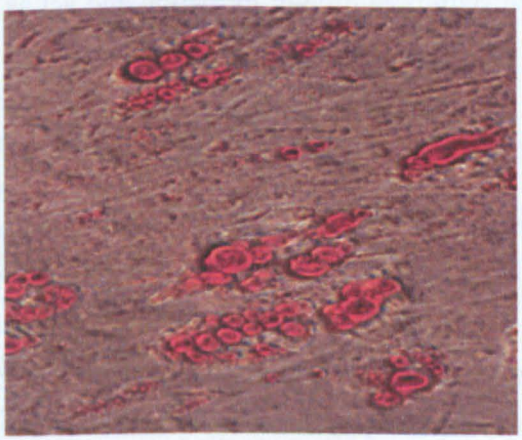

Figure 51. 4 weeks differentiation of PCOS adipocytes with Oil Red O Staining. 70% of the cultures have filled with small lipid droplet. Larger droplets are beginning to form. The Oil Red O staining shows that more adipocytes are maturing with increased lipid droplet size and number when compared to non-PCOS cells at the same time course. 10x magnification.

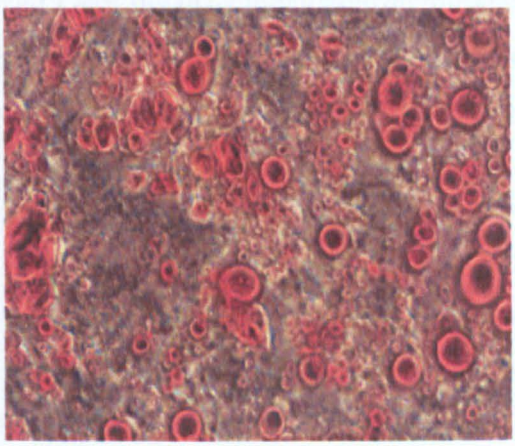

Figure 52. 9 weeks differentiation of PCOS adipocytes with Oil Red O Staining. 80% of the cultures have filled with large lipid droplet. When compared with non-PCOS adipocytes at the same time course the droplets size is larger and numbers increased. 10x magnification.

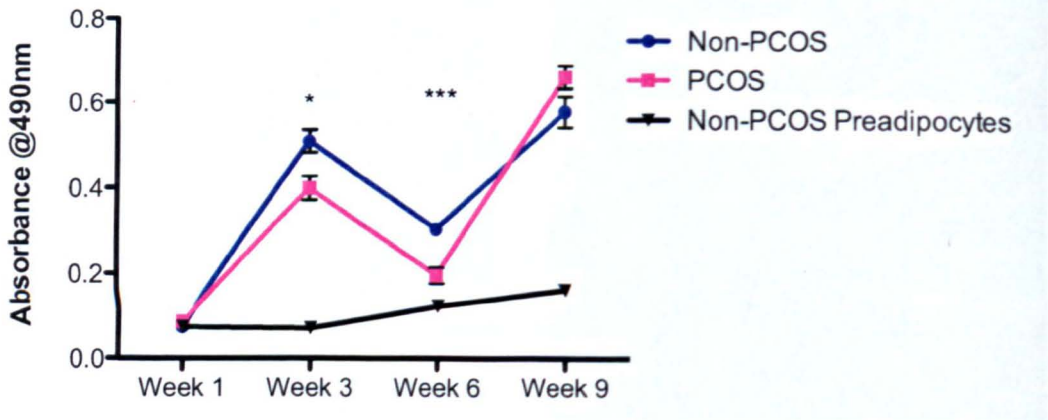

Figure 53. Non-PCOS and PCOS differentiation (9 weeks n=6 vs. 6). Non-PCOS and PCOS preadipocytes were differentiated over 9 weeks. Media was removed every 3 weeks and cells stained with Oil Red O. Oil Red O were removed and absorbance measured 490nnm. Non-PCOS preadipocytes were cultured alongside and differentiation not initiated for control.

By using Oil Red O staining of cell cultures at 3 week time courses we were able to measure differentiation. The oil Red O would stain lipid droplet formation and act as measure of maturation. This could then be measured through removal of the remaining Oil Red O and absorbance measured again (*see 3.2.2.1*) Similar to the morphological findings, a comparison of 9 week differentiation of non-PCOS and PCOS adipocytes showed significant variations across cultures. **Figure 53** shows that by week 3 a significant difference in differentiation occurs (Mean ± S.E.M. 0.1098 ± 0.03933 *P<0.0106). Although a decrease in absorbance is seen by week 6 significant variations between the cultures is maintained until week 9 where the highest level of maturation is reached. This is seen statistically to be a similar level over both non-PCOS and PCOS groups with no significance between the absorbance found.

4.5 Proliferation in non-PCOS and PCOS adipocytes.

In order to examine the effects hormonal treatments may have on adipocyte proliferation within both non-PCOS and PCOS preadipocytes, it was necessary to establish a growth curve under stable conditions. In order to do this, MTS assays (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) were used to colormetrically assess proliferation and cell viability in cells derived from non-PCOS cells and PCOS patients.

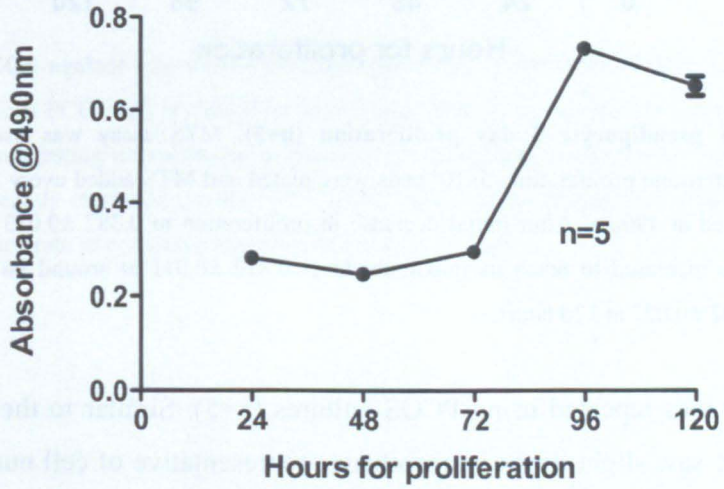

Figure 54 Non-PCOS preadipocyte 5 day proliferation (n=5). MTS assay was used on Non-PCOS preadipocytes to determine proliferation. $5x10^3$ cells were plated and MTS added every 24 hours with absorbance measured at 490nm. After initial decrease in proliferation following 48 hours cell numbers increased to reach its maximum level at around 96 hours before levelling off at 120 hours.

Non-PCOS cells were plated (n=5 / $5x10^3$ cells) and MTS absorbance measured every 24hrs from day 1. Results were measured against a standard. Once plated absorbance showed an initial decrease. Rapid growth then occurred to its peak cell density of around 0.733 ± 0.013 represented by absorbance at 96 hours. At this stage cell numbers decreased to 0.652 OD ± 0.20 (*see* **Figure 54**.)

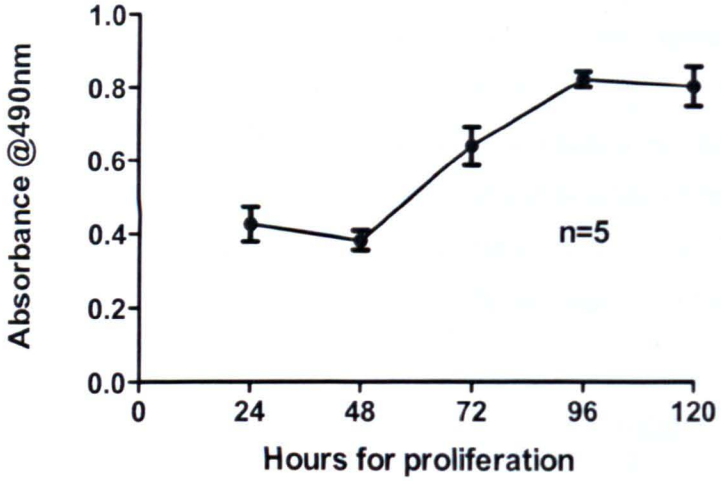

Figure 55. PCOS preadipocyte 5 day proliferation (n=5). MTS assay was used on PCOS preadipocytes to determine proliferation. $5x10^3$ cells were plated and MTS added every 24 hours with absorbance measured at 490nm. After initial decrease in proliferation to 0.382 ±0.013 following 48 hours cell numbers increased to reach its maximum level 0.822 ±0.011 at around 96 hours before levelling off at 0.802 ±0.027 at 120 hours.

The MTS assay was repeated using PCOS cultures (n=5). Similar to the non-PCOS growth curve we saw slight drops in absorbance representative of cell numbers at 48 hours followed by a sharp rise in proliferation at 72 and 96 hours to a maximum 0.822 ±0.011 OD (*see* **Figure 55**). By 120 hours the cultures had settled at 100% confluence at around 0.822 ±0.0110.027 OD. Cross-examination of non-PCOS against PCOS growth curves showed no significant difference in plating density or final cell yield. However 72 hours showed a significant rise in proliferation within the PCOS cells (P=0.1035, 3332 ± 368)(**Figure 56**).

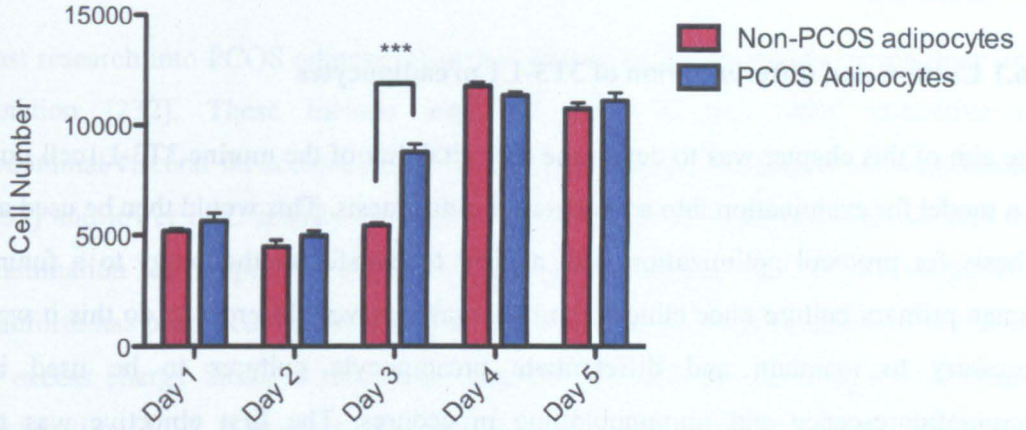

Figure 56. PCOS against non-PCOS preadipocyte 5-day proliferation (n=5). Column analysis of non-PCOS against PCOS cell proliferation over 5 days showed no variation in cell numbers at seeding or final yield suggesting no variations in preadipocyte morphology. Results are mean ± SEM, Day 3 showed significantly increased growth (***P<0.001) following examination with unpaired *t-test*. This suggest increased rate in proliferation within PCOS preadipocytes.

4.6 Discussion

4.6.1 Culture and differentiation of 3T3-L1 preadipocytes

The aim of this chapter was to determine the suitability of the murine 3T3-L1cell line as a model for examination into adipocyte steroidogenesis. This would then be used as a basis for protocol optimization with a view to transfer methodology to a future human primary culture once ethical approval was received. In order to do this it was necessary to maintain and differentiate preadipocyte cultures to be used in immunofluorescence and immunoblotting procedures. The first objective was to establish and differentiate 3T3-L1 preadipocyte cultures. This has been a long established protocol and although the cell line proved to be proliferative and achieved confluence within T75 flasks over 8-9 days, initial difficulty was found in using the protocol as described in Chawla *et al.*, (1994)[254]. Following cyclic changes between maintenance media and stimulatory media, cultures showed partial differentiation but were unable to survive. Successful differentiation occurred utilizing the protocol as described by Chen *et al.*, (1997) using a single stimulatory step with increased insulin, dexamethasone and the addition of IBMX [253]. The combination of dexamethasone and IBMX, a phosphodiesterase inhibitor, acted as an inducing agent, which has been suggested to either trigger or accelerate differentiation [249]. Differentiation did occur spontaneously prior to dilution of initiation media in some cases as seen in **Figure 36** suggesting this media may therefore act as an accelerant of differentiation. Partial differentiation was achieved after 4 weeks with no adipocytes retaining preadipocyte cobblestone or elongated morphology. Mature adipocyte formation was measured on enlarged lipid droplet formation, which did not occur until week 6 and did not develop beyond 80% of the cell culture. By continuing the differentiation procedure beyond 6 weeks, eventual de-differentiation occurred followed quickly by cell death. This maybe as a result of cytokine release as described by Prins *et al.*, 1997 [255, 256] whereby increased adipocity signals increased tumour necrosis factor-alpha (TNF-α) this subsequently leads to de-differentiation as a means of regulation and results in apoptosis [255]. This dynamic process therefore requires careful monitoring in order to maintain adipocyte cultured at the desired stage of differentiation.

4.6.2 Differentiation in non-PCOS and PCOS adipocytes

Past research into PCOS adipose tissue has shown variations within morphology and function [232]. These include increased waist to hip ratios indicative of abdominal/visceral fat accumulation, enlarged adipocytes, reduced serum adiponectin [257] and lower LPL activity [232, 258]. Although there is limited research into the examination of adipocyte differentiation across normal and PCOS adipocytes Danforth has proposed that an inability for preadipocytes to differentiation as a result of excess energy intake within obese patients may account for observed metabolic dysfunction [259]. If this were the case it would lead to redistribution of fat depots where ectopic distribution has been shown to encourage insulin resistance. This hypothesis has been supported by Yang who on examination of adipocytes from obese and non-obese subjects showed those with insulin resistance had reduced expression of genes associated with differentiation such as aP2 and adiponectin [260]. In addition to this, Gupta *et al.*, have demonstrated reduction in differentiation as well as lipid accumulation in normal human adipocytes when exposed to dihydrotestosterone typically found to be increased within PCOS women [261].

Our results suggest that samples taken from PCOS women have reduced rates of differentiation across a range of BMIs from lean to obese. As all samples were matched across non-PCOS and PCOS groups the significant variation seen is supportive of these hypothesis. Findings also showed that although a decrease in differentiation occurred at week 6, the variation between groups remained significantly different supporting a difference within rates between groups. This is further confounded by the point at which both groups of adipocytes reach an equal level of maturity. The dip in lipid droplet formation at week 6 may be explained by an inadequacy of the protocol chosen. Typically oil red O staining is used as a marker of the characteristics of adipocytes rather than a comparative measure of differentiation. Although 6 samples per group were used in duplicate, cells were seeded in 4 different plates to allow examination of a specific plate at a specific time course i.e. week 3, 6, and 9. As adipocyte cells are particularly delicate during maturation especially due to lipid buoyancy, the required washing and treatments involved within the protocol may have affected the results. The suitability of this method for this experiment would therefore require comparison to an alternate measure of maturation and with further

work biochemical examination may support this possibly by examining glycerol 3-phosphate dehydrogenase (GPDH) levels [262]. Studies of *in vitro* cultures of both animal and human have however shown that differentiating adipocytes can undergo a stage of dedifferentiation and still fully develop into mature adipocytes with no effect on the characteristic seen within normal adipocyte maturation [263, 264].

4.6.3 Proliferation in non-PCOS and PCOS adipocytes.

Previous research has shown hypertrophy within PCOS abdominal SC mature adipocytes independent of BMI [185, 232]. Examination of preadipocytes morphology showed no differences between sample groups at this stage of differentiation. This may suggest variation occurs during adipocyte development.

This was further supported through equal levels of confluence reached within our proliferation assays (**Figure 54**, **Figure 55**). Both culture groups stabilized at around absorbance 0.7 OD (approx. 11,000 cells). Assuming both cell types behaved similarly avoiding stacking / clumping during proliferation, then cell morphology would dictate the space available and the subsequent yield.

Studies into non-obese PCOS women have shown marked decreases within catecholamine-induced lipolysis in SC abdominal adipocytes suggestive of resistance [235, 265]. If this were the case it would prevent mobilization of triglycerides increasing lipid droplet storage, which accounts for 95% of mature adipocytes and dictates the subsequent size of the cell. This would also explain why variations in PCOS morphology only occur following differentiation.

Separate examination of both sample groups allowed us to see similar growth curves with slight decreases in proliferation following seeding typically found in plating density due to acclimatization. Increased growth was seen by day 3 reaching the optimum level of confluence for the experiment by day 4 and showing a slight decrease in number to stabilize by day 5,6 and 7. This is typically due to depletion of growth factors, space available and nutrients; all contributing to an inability to sustain increased cellular yield. Rapid growth can also often lead to initial clumping and stacking of cells. Depending on the cell type some cells cannot survive in this state and numbers decline to a more stable level. Unpaired *t-test* analysis of each day between non-PCOS and PCOS showed no variation within sample groups with the

exception of day 3. At this point significant variation was seen suggesting hyperplastic behaviour within PCOS preadipocytes as theorised within PCOS ovarian theca cells [266, 267]. This result may be considered a biological observation, however it may be attributed to the sample numbers examined and therefore statistical limitations. Mature adipocytes proliferate at a very slow rate and controversy exists as to whether this ability exists at all [223, 268]. Increased adipose tissue is therefore typically attributed to hypertrophic expansion of existing adipocytes. Although we are aware that PCOS mature adipocytes show increases in hypertrophy, this has been linked to increased pro-inflammatory markers with chemokine levels (MCP-1 and MIP-1) having been shown to correlate with BMI within PCOS women [269-271]. Although this hypothesis may explain the association with obesity and PCOS, it would require further examination into why this increase in pro-inflammatory mediators would not initiate cell death typically found in non-PCOS adipocytes and in turn would reduce cell numbers. Hyperplastic PCOS preadipocytes may therefore go some way to explaining the pathogenesis of associated obesity within the condition. A combination of preadipocyte hyperplasia and possible decreased triglyceride mobilization within PCOS may therefore offer a link between obesity and PCOS.

In summary, this chapter suggests that variations exist in the metabolic activity of *in-vitro* SC adipocytes from PCOS patients.

5.0 CHARACTERISATION OF THE STEROIDOGENIC PATHWAY IN 3T3-L1 CELLS AND ADIPOCYTES OF WOMEN WITH AND WITHOUT PCOS

5.1 Introduction

Studies as early as 1987 have shown adipose to be a major site of sex steroid metabolism with interest arising from positive correlation to increased oestrogen and androgen levels found with increases in weight gain [207, 272]. The identification and characterisation of leptin in 1994 [225] further refuted the myth that adipose was a passive reservoir for energy storage. It also created the possibility of a loop system theory, whereby brain and peripheral communication were involved in fuel and body weight homeostasis. Leptin was secreted and seen to effect the hypothalamic neuropeptide Y (NPY) allowing energy regulation [193]. Since then, adipose function has been widely examined with adipocytes shown to express and secrete a variety of bioactive peptides that include C-reactive protein (CRP), angiotensinogen, leptin and resistin, that act in both an autocrine and paracrine manner [224, 249, 252]. Additionally, it has been shown that adipocytes express various factors and receptors that mediate their autocrine responses. Receptors have also been determined with regard to many traditional hormonal signals leading to subsequent activation of intracellular pathways and supporting the communicative abilities of adipocytes (see **Table 6**) [226-228]. Leptin is synthesised by adipocytes and is believed to act in both an autocrine and paracrine manner. The signal pathways regulated by leptin are now known to be diverse including both cytokine and growth factor receptor signalling [273]. It has also been seen to act through its own receptor in lipolytic regulation within adipocytes, as well as its action following secretion, in other organs including the hypothalamus through which it regulates satiety [226].

Research has begun to establish the major involvement adipose tissue has in the regulation of levels and bioactivity of sex steroids. This can be seen within adipocyte conversion of peripheral androstenedione to more potent androgens, and in the synthesis of oestrogen, with adipocyte biosynthesis seen as the main contributor to physiological oestrogen levels in postmenopausal women [224, 274, 275].

Examination into the steroidogenic pathway involved in oestrogen conversion/synthesis has shown the involvement of cytochrome P450-dependent aromatase in precursor androstenedione transformation to oestrone, and it is believed that changes in localised levels of these hormones may be involved in sex related fat deposition [208].

Adipogenesis and subsequent fat deposition, is dependent on preadipocyte precursor development and induction via transcriptional activation from key factors including adipsine, aP2, LPL as well as a variety of hormones including insulin, IGF-1 and glucocorticoids. (5, 6, 7). Although the role of sex steroids in this process is yet to be fully understood, studies by Dieudonne *et al.*, 2002, have shown that oestrogen has a preadipogenic effect whereas androgens show a more antiadipogenic effect on the production of SC fat in 3T3-L1 cells [276].

Factors secreted by adipocytes for autocrine	**Reference**
Leptin	Zhang *et al.*, 1994
Agouti	Bultman *et al.*, 1992
Sex Steroids (progesterone, oestrogen,	Bouloumie *et al.*, 1994
Cytokines, tumour necrosis factor (TNFα)	Hotamisligil *et al.*, 1993
Interleukin-6 (IL-6)	Fried *et al.*, 1998
Free fatty acids e.g.	Coppack *et al.*, 1994
Lipoprotein lipase	Fried and DiGirolamo *et al.*,
Apoprotein E (Apo E)	Zechner *et al.*, 1991
Insulin-like growth factor-1 (IGF-1)	Doglio *et al.*, 1987

Table 6. Receptor expression in adipocytes. Studies have shown a wide array of receptors expressed within sub-cutaneous adipocytes that would allow signalling to occur through hormonal influence [226-228].

It is well recognised that androgens are produced in both the ovaries and the adrenal glands [8, 277, 278]. However, few studies have examined the capabilities of peripheral tissues such as the skin, liver and adipose tissue to convert weak circulatory androgens into stronger androgens or oestrogen. Investigation into adipose steroid conversion has shown 15 steroidogenic enzymes exist within adipose tissue including aromatase, 3β-hydroxysteroid dehydrogenase (HSD) type 1 (31), 11β-hydroxysteroid dehydrogenase type 1 and 2 (35), 5α-reductase (36) and 17β-HSD types 2,3 and 5

[210]. Many of these are also present in the ovary. Based on these findings, the capacity for peripheral tissue to synthesize and inactivate androgens is feasible.

Our findings shown in Chapter 4, suggested that variations in metabolic behaviour may exist between non-PCOS and PCOS SC adipocytes. We therefore further examined comparisons between the two sample groups with focus on PCOS associated dysfunctions. In the present study we therefore aimed to show the viability of localized androgen synthesis through the presence of the steroid-converting enzyme 17α-hydroxylase (CYP17) and the progesterone precursor. Our rationale was based on the androgen synthetic pathway found within ovarian steroidogeneisis and how the presence of the precursors and key enzymes necessary for thecal steroid metabolism, if determined present within adipocytes, would allow androgen synthesis. This theory was examined within preadipocytes and following differentiation under controlled conditions. Subcutaneous adipose biopsies of both non and PCOS adipose samples were used in primary culture as previously determined to be more steroidogenically active than visceral samples [209]. SC biopsies were also used due to the occurrence of abdominal obesity typically associated with PCOS and suggested metabolic dysfunction, which has been hypothesised (*see 1.10*). Examination of a localized source of androgen production is therefore of clinical relevance due to the association of obesity and increased androgen secretion, asymptomatic of PCOS.

5.2 Objectives

i. Determine the presence of 17α-hydroxylase within preadipocytes and mature 3T3-L1 cultures.

ii. Determine the presence of the steroidogenic enzyme 17α-hydroxylase within adipocytes obtained from non-PCOS and PCOS patients.

iii. To examine hormonal secretion of androstenedione and progesterone in non-PCOS and PCOS adipocytes *in vitro*.

5.3 Method

Readily established 3T3-L1 and primary human adipocyte cultures (non-PCOS and PCOS) were used as either preadipocytes or mature adipocytes (*see 3.2.1/3.2.2*).

The expression of CYP17 in 3T3-L1 cells was investigated by immunofluorescence (IF)(*see 3.5*). 3T3-L1 cultures where used prior to receiving ethical approval of the human model. This allowed validation of the chosen protocol whilst identifying its suitability if required for any further work. From this, optimization of protocols for use within our human primary cultures was achieved.

Sparsely cultured ($5x10^4$) 3T3-L1 preadipocytes were fixed with acetone methanol, incubated with anti-CYP17 antibody (1:50 Abgent AP7879c) and secondary FITC, and washed. These were then examined under a fluorescent microscope (*see method 3.2.1*).

Chinese hamster ovary (CHO) cells at passage 12 were used as a positive control for CYP17 immunofluorescence [192, 279]. β-Actin antibody (Abcam Ab8227) was also used as a positive control for the protocol. Negative controls included leucocytes prepared using the same protocol [280]. Preadipocytes, CHO and leukocytes were also incubated in the absence of primary antibody.

In order to support the expression of CYP17 within our adipocyte cultures we used semi-quantitation of proteins by western blotting (*see 3.6*). The BCA technique along with Ponceau S staining and β-Actin probe was used on preadipocyte lysate harvests and subsequent immunoblot to confirm equal protein loading (*see 3.6.3*). Nitrocellulose blots were probed using the polyclonal CYP17a1 antibody (1:50 SantaCruz Sc46085) Following optimisation of protocol; exposure at 640sec was examined for band exposure at 57 kDa band which is the predicted molecular weight of CYP17

5.4 Results

5.4.1 Expression of the steroidogenic enzyme CYP17 within 3T3-L1 cells using immunofluorescence

Preadipocytes were cultured to approximately 50% confluence to allow individual cell morphology to be seen under fluorescence (**Figure 57**) and phase contrast (**Figure 58**) microscopy. Negative controls (leucocyte culture and secondary antibody alone) showed no fluorescence indicative of no CYP17 expression. CHO cultures showed fluorescence positive for CYP17 (**Figure 59**). β-Actin probing showed fluorescence substantiating the protocol used (**Figure 57**) based on the widespread expression of this housekeeping protein.

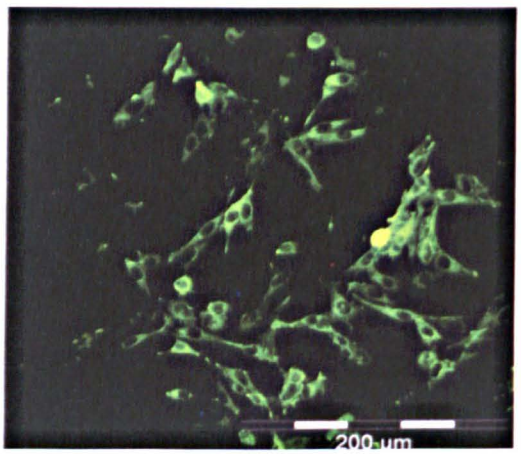

Figure 57. Immunofluorescence of 3T3-L1 adipocytes. 3T3-L1 preadipocytes stained with β-Actin and FITC conjugated secondary antibody. β-Actin was used as a positive control due its occurrence as a cellular component of the cytoskeleton.

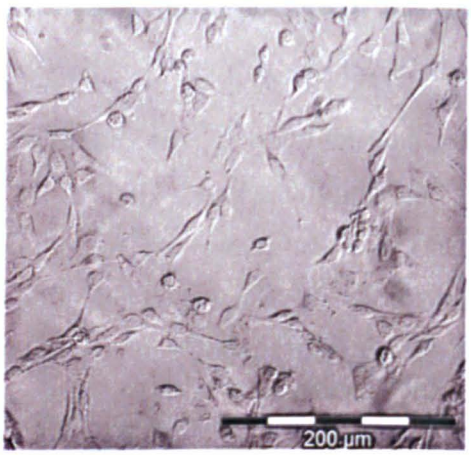

Figure 58. Phase contrast 3T3-L1 adipocytes. 3T3-L1 pre adipocytes shown in phase contrast. Used to see cell morphology as a comparison for successful fluorescence via β-Actin.

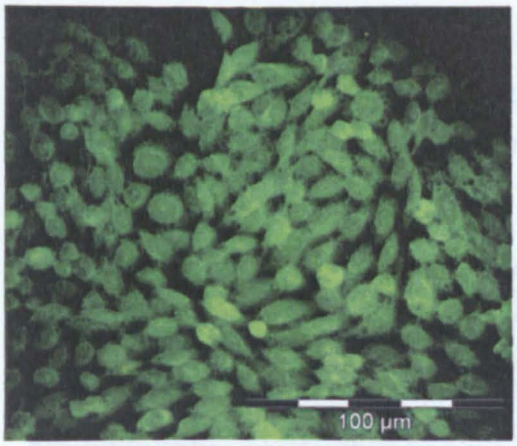

Figure 59. Immunofluorescence of Chinese hamster ovary cells (CHO). CHO cells passage 12 stained with CYP17 and FITC conjugated secondary antibody. CHO cells were used as a positive control showing the expression of CYP17 within the cytosol.

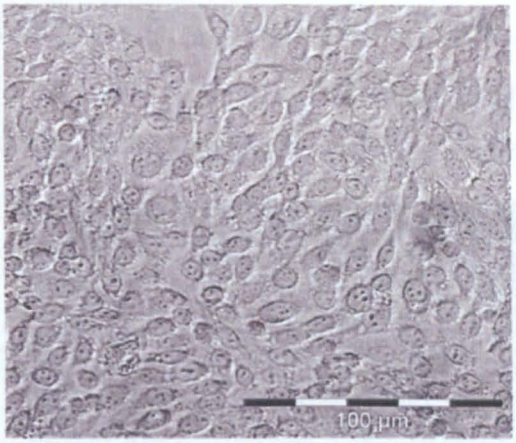

Figure 60. Phase contrast CHO. CHO cells shown in phase contrast. Showing cell morphology and comparison for immunofluorescence images.

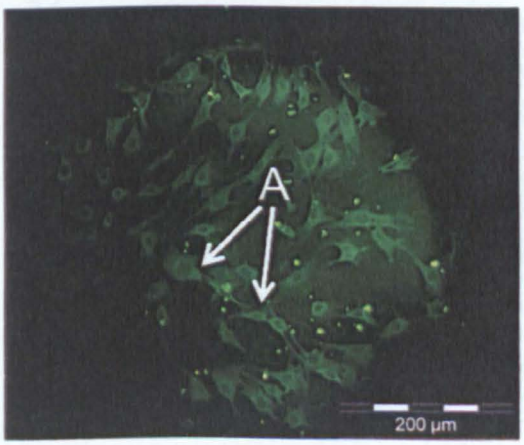

Figure 61. Immunofluorescence of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes 16th passage stained with CYP17 and FITC conjugated anti-rabbit secondary antibody. Fluorescence is localised in the perinuclear region and in the cytosol. **A)** Concentrated perinuclear CYP17 staining can be seen.

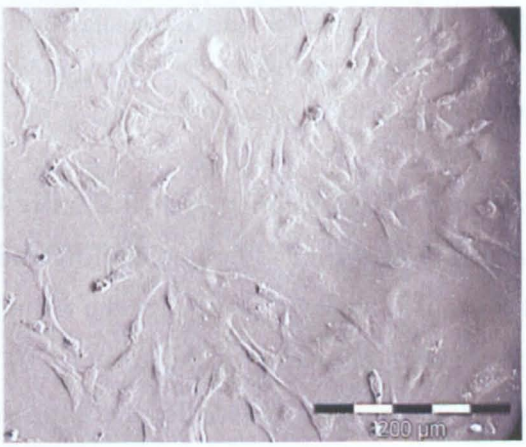

Figure 62. Phase contrast image 3T3-L1 preadipocytes. 3T3-L1 preadipocytes grown to 40-50% confluence to allow imaging of cell morphology.

Figure 63 shows that CYP17 was predominantly expressed within the cytoplasm with abundant perinuclear localisation. Mature adipocyte cell morphology was clearly seen

with rounding of the preadipocytes following development of lipid droplets, which encompassed the majority of the cytosol. These results showed CYP17 localisation within the 3T3-L1 preadipocytes cytoplasm prior to differentiation.

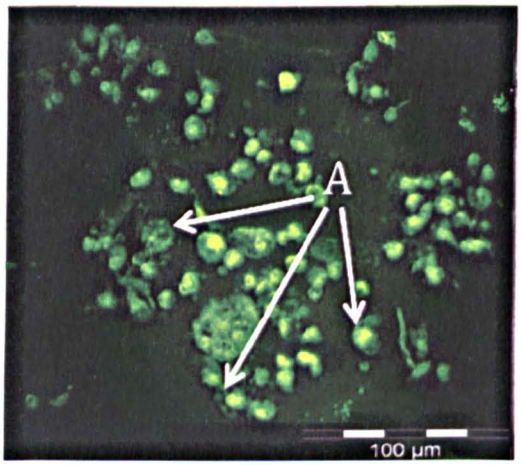

Figure 63. Immunofluorescence of 3T3-L1 mature adipocytes. 3T3-L1 mature adipocytes following 6 weeks differentiation stained with CYP17 and FITC conjugated secondary antibody. **(A)** Fluorescence can be seen surrounding lipid droplet formation and within the cytosol. Some staining occurs across cellular debris.

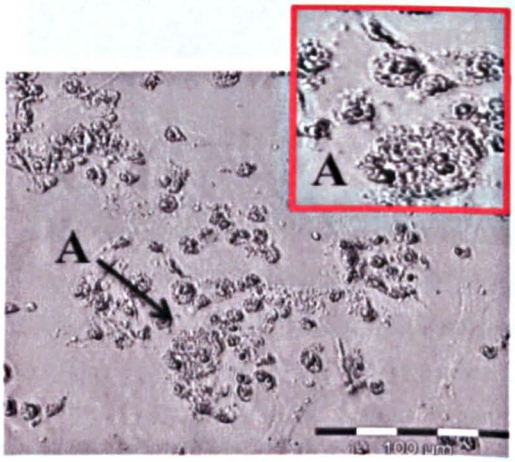

Figure 64. Phase contrast 3T3-L1 mature adipocytes. 3T3-L1 mature adipocytes shown in phase contrast following 6 weeks differentiation. Lipid formation following differentiation can be seen within cells and raised across the z-axis due to buoyancy.

The perinuclear staining seen in **Figure 63** also suggests CYP17 is present at this stage of development. Following differentiation the same protocol was used for 3T3-L1 mature adipocytes. Although the technique was not as robust due to the fragility of adipocytes, **Figure 63** shows that CYP17 antibody did produce fluorescence. The variation in z-axis caused by the large size of these cells made focusing difficult, however fluorescence can be seen surrounding the lipid droplets and in the cytoplasm (**Figure 63 (A)**) when compared to phase contrast (**Figure 64**). Fluorescence was also observed within some lipid droplets. This may be suggestive of rupturing during acetone-methanol fixation, or presence of CYP17. This may also be a result of disparity within the z-axis or general culture debris. These results show the expression of CYP17 at both pre and mature stages of differentiation.

5.4.2 Western blot analysis of CYP17 expression in 3T3-L1 adipocytes

Western blot analysis of CYP17 expression in 3T3-L1 adipocytes both preadipocytes and mature, showed a predicted band at 57kDa suggesting the presence of CYP17 in both pre and mature 3T3-L1 adipocytes. All blots were exposed for 640sec **Figure 65** using equipment and conditions as described in *3.6.4*.

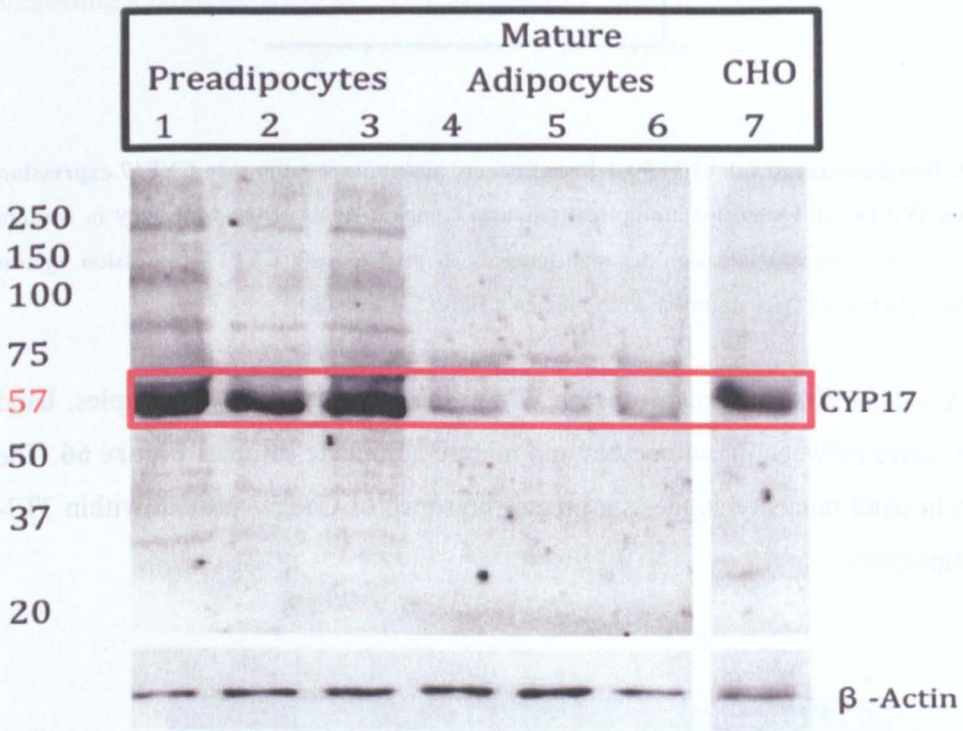

Figure 65. Shows Western blot CYP17 expression of 3T3-L1 preadipocyte (lane 1-3) and mature adipocyte (lane 4-6) lysates. Preadipocyte (lanes 1-3) and mature adipocyte lysates (lane 4-6) probed using CYP17a1 antibody against positive control cell line Chinese hamster ovaries (CHO lane 7). shows bands at 57kDa, which were more prominent in preadipocyte samples. The blot was then stripped and re-probed using β-Actin to control for protein loading.

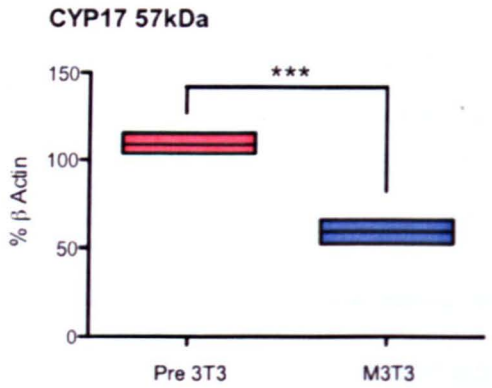

Figure 66. Densitometry graph of 3T3-L1 Preadipocyte and mature adipocyte CYP17 expression taken from WB (n=3). Using β-Actin normalisation to compare levels of band intensity in western blot CYP17 expression showed significant differences in preadipocyte CYP17 expression against mature adipocyte lysates Data summarised as median ± IQR (*** P<0.001).

Although a detectable band can be seen within the mature adipocyte samples, band intensity varies between preadipocytes and mature adipocyte samples **Figure 66**. The variation in band intensity suggests a greater presence of CYP17 protein within 3T3-L1 preadipocytes.

CYP17	n	MEDIAN ± IQR	*P.*
Pre Adipocytes	3	107.8(103.7-115.5)	0.007
Mature Adipocytes	3	59.46(52.25-66.02)	*** Sig

Table 7. Summarising the densitometry representative of average expression of CYP17 in pre and mature 3T3-L1 samples. A Significant difference was seen in expression of CYP17 (Mwt 57 kDA) between the samples with preadipocytes showing the greater protein yield. Data summarised as median ± IQR (*** P<0.001)

Examination of our results shows β-Actin did not vary between samples and from this we can assume equal protein loading across all lanes. β-Actin was therefore used for normalisation and semi-quantitative expression. The box graph seen in **Figure 66 (B)** shows the percentage intensity against the β-actin control and variation between samples can be seen. There is a significant difference in CYP17 expression across the two samples with preadipocyte 3T3-L1 showing the greatest expression and suggesting a large decrease in CYP17 expression occurs during maturation.

5.4.3 Expression of the steroidogenic enzyme CYP17 within primary human adipocytes

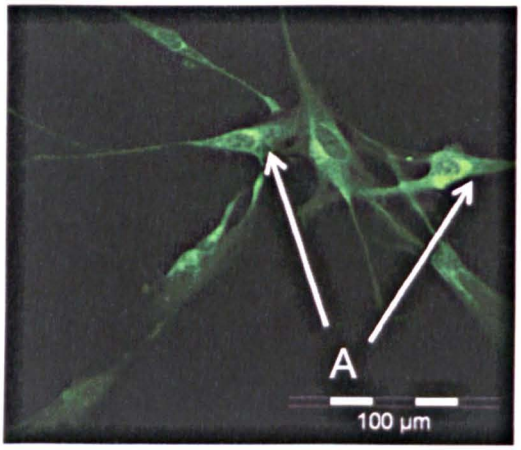

Figure 67. Immunofluorescence of human preadipocytes. Human preadipocytes stained with CYP17 and FITC conjugated anti-rabbit secondary antibody. CYP17 expression is located within the cytoplasm. (A) Concentrated perinuclear CYP17 staining can be seen.

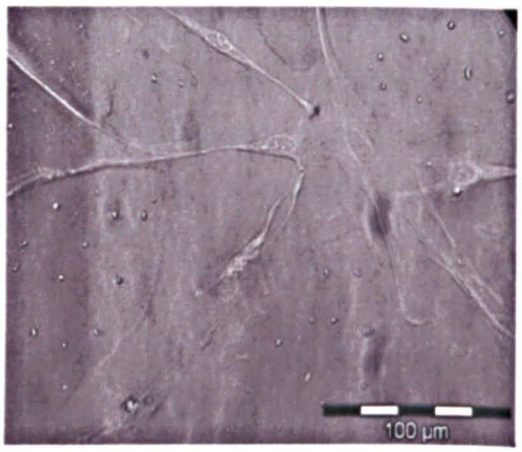

Figure 68. Phase contrast human preadipocytes. Human preadipocytes grown to 40-50% confluence to allow imaging of cell morphology.

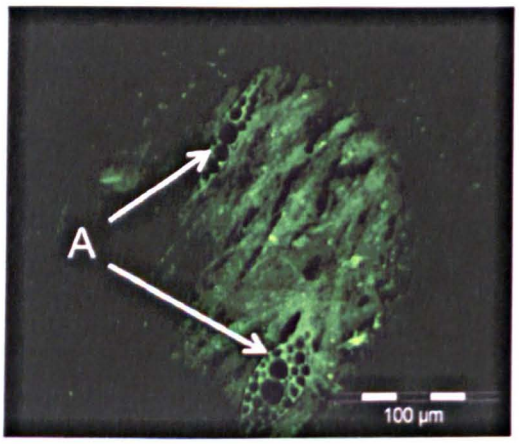

Figure 69. Immunofluorescence of human mature adipocytes. Mature human adipocytes stained with CYP17 and FITC conjugated secondary antibody. (A) Fluorescence can be seen surrounding lipid droplet formation and within the cytosol.

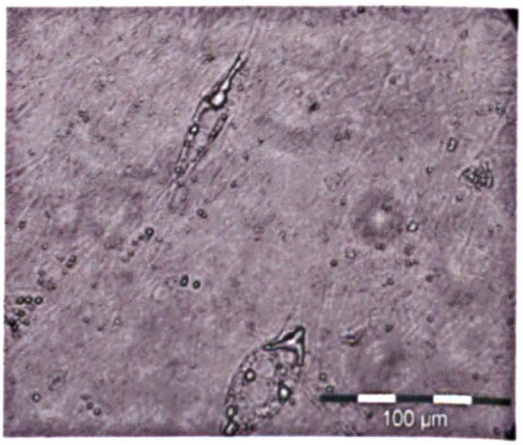

Figure 70. Phase contrast human mature adipocytes. Human mature adipocytes shown in phase contrast. Lipid formation following differentiation can be seen within cells and raised across the z-axis due to buoyancy.

Examination of the presence of CYP17 within pre and mature adipocytes of our human cultures showed almost identical results as seen in the mouse 3T3-L1 cells.

Preadipocytes showed good fluorescence when probed with CYP17 antibody with areas of fluorescence localized to the cytosol. Once again we found peri-nuclear staining **Figure 67**. All results were supported through comparative CHO cultures, negative control leucocyte cultures (*see 3.5.1*) and in the absence of primary antibody (not shown). Using the 3T3-L1 cell line allowed us to optimize the protocol and we found that using the acetone-methanol method of fixation rather than the published paraformaldehyde [252], allowed better results to be seen within our mature adipocyte cultures and showed better representation of the formation of enlarged lipid droplets **Figure 69** (A).

5.4.4 Western blotting analysis of CYP17 expression within human adipocytes

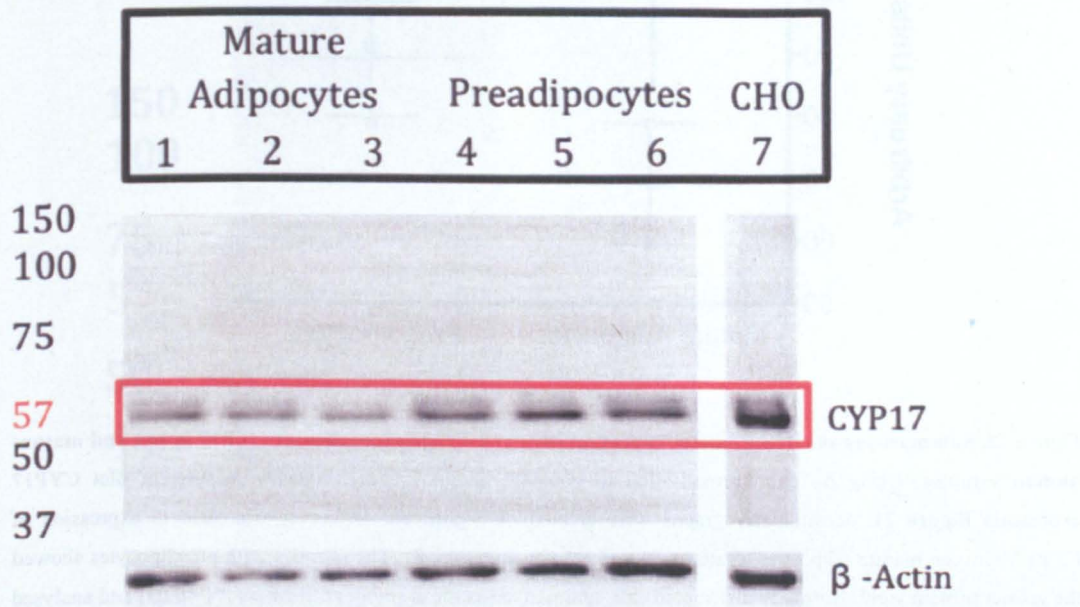

Figure 71. Shows Western blot CYP17 expression of human preadipocyte (lane 1-3) and mature adipocyte (lane 4-6) lysates. Preadipocyte (n=3 lanes 1-3) and mature adipocyte lysates (n=3 lane 4-6) probed using CYP17a1 antibody. Exposure at 640sec shows bands at 57kDa, which were more predominant in preadipocyte samples. The blot was then stripped and reprobed using β-Actin to allow for loading control. (B) Shows the box plot for CYP17 in both pre and mature lysates normalised to match β-actin expression.

A western blot analysis was run using lysates harvested from primary human pre and mature adipocyte cultures (**Figure 71** n=3 *vs.*3). After using various P450C17/CYP17 antibodies we found the best results were achieved using CYP17a1 (ab7879c) antibody, allowing for a relatively clean specific blot at reasonably low exposure. This

antibody was kept constant throughout all our human sample work. **Figure 71** showed predicted bands at around the 57kDa mark in both preadipocyte and mature adipocyte samples. Mature adipocytes (lanes 1-3) showed consistently less band intensity than that of the preadipocytes as were seen within our animal cells. Control lysates from CHO cultures were used to validate (**Figure 71**, lane 7). All lanes were also normalized using β-actin antibody.

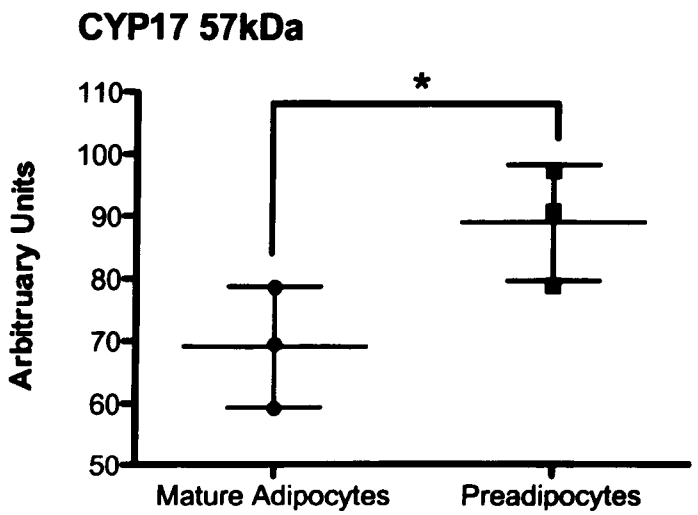

Figure 72. Summarising the densitometry representative of average expression of CYP17 in pre and mature human samples. Using β-Actin normalisation to compare levels of band intensity in western blot CYP17 expression **Figure 71**, densitometry graphs were created. A Significant difference was seen in expression of CYP17 between mature adipocyte lysate (n=3) and preadipocytes (n=3). The samples with preadipocytes showed the greater protein yield. Normally distributed data summarised as mean (range) / SEM (±) (*P<0.05) and analysed using unpaired *t-test*. Results are mean ± SEM

5.4.5 Western blotting analysis of CYP17 expression in non-PCOS and PCOS human adipocytes

Non-PCOS and PCOS lysates from mature adipocytes were examined using the western blot technique for expression of CYP17 (non-PCOS n=4 *vs.* PCOS n=3) **Figure 73** shows expression at 57kDa was seen in both mature adipocyte lysates taken from non-PCOS and those taken from PCOS lysates, suggestive of the presence of CYP17. All lanes were normalized against β–Actin.

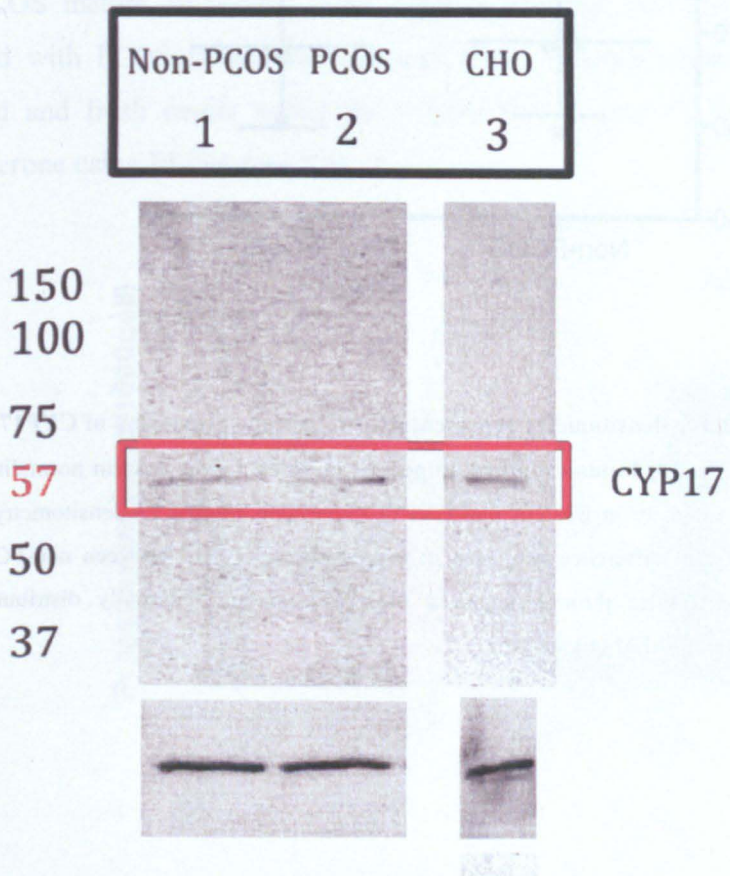

Figure 73. Shows Western blot analysis of CYP17 expression of mature human adipocytes from non-PCOS (n=4) and PCOS lysates (n=3) (lane 1-2). Expression at 57kDa was seen in all lanes including the CHO positive control (lane 3) suggestive of CYP17 presence. No variation in band intensity was seen between non and PCOS samples. β-Actin was used as a loading/positive control.

Figure 73 showed no variation in band intensity between non-PCOS and PCOS mature adipocyte lysates. **Figure 74** shows no significant variation when examining the densitometry results in western blot exposures between non-PCOS and PCOS

mature adipocyte lysates (n=4 *vs.* n=3). This would suggest no difference in CYP17 expression occurs across the two groups.

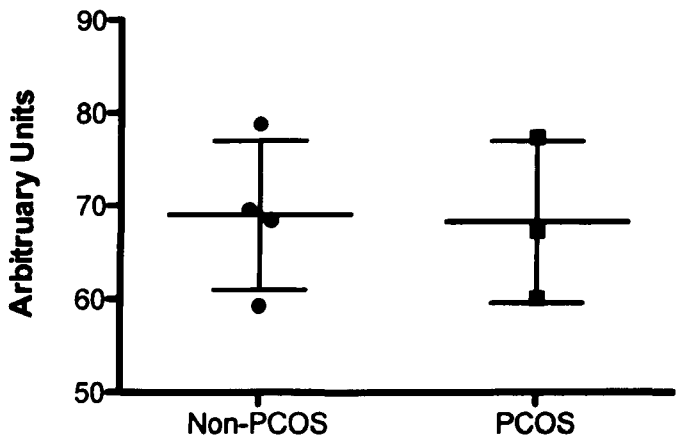

Figure 74. Summarising the densitometry representative of average expression of CYP17 in non-PCOS (n=3) and PCOS (n=4) human mature adipocyte samples. Using β-Actin normalisation to compare levels of band intensity in western blot CYP17 expression **Figure 73** densitometry graphs were created. No Significant difference was seen in expression of CYP17 between non-PCOS and PCOS mature adipocyte lysates through unpaired *t-test* examination. Normally distributed data summarised as mean (range) / SEM (±).

5.4.6 Hormone secretion in mature adipocytes

Following careful control of media and environmental conditions from biopsy, culture development and maturation (*see 3.2*); mature adipocytes were assayed and secretion of various hormones including progesterone, androstenedione and testosterone were measured using ELISA.

5.4.6.1 Adipocyte progesterone secretion.

Non-PCOS mature adipocytes were cultured (passage 2-3 n=5) from biopsy and matched with PCOS sample for age and BMI. Following maturation, media was changed and fresh media added for 3 days before removal and examination for progesterone using ELISA (*see 3.6*).

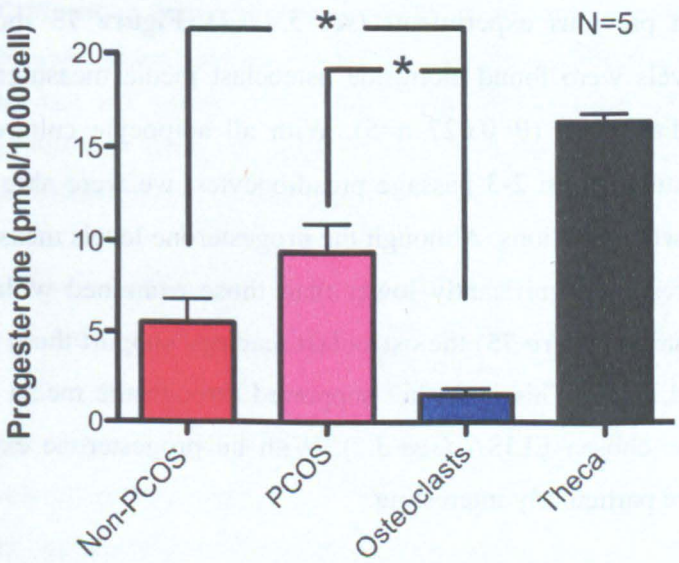

Figure 75. Progesterone secretion by non-PCOS and PCOS mature adipocytes (n=5) over 3 days. PCOS and non-PCOS progesterone secretion was measured and examined against negative/positive control using one-way ANOVA. Osteoclast progesterone secretion was used as a negative marker and theca progesterone secretion used as a positive marker. In both non-PCOS and PCOS samples significance was seen *P<0.05. No significance was seen between non-PCOS and PCOS progesterone secretion. Results are mean ± SEM.

Figure 75. shows progesterone was measured within adipocyte media following 3 days maintenance (Mean 5.5 ± 1.29pmol/1000 cells). A significant difference was seen between non-PCOS and osteoclast samples with an increase in progesterone

levels in the non-PCOS samples (P = 0.018). Theca cells were also used as a positive marker of progesterone secretion as well as an intra control throughout all the ELISA techniques performed. Again 3 day untreated media was removed from theca cultures and results normalised to pmol/1000 cells. Results seen in **Figure 75** showed progesterone secretion to be 3 times that found within the adipocyte media (mean 16.37 ± 0.44pmol/1000).

5.4.6.2 Comparison of non-PCOS and PCOS adipocyte progesterone secretion.

Progesterone secretion was also measured within media harvested from PCOS samples (**Figure 75**) examination of media taken from PCOS cultures maintained for 3 days was done using the same protocol.

Progesterone levels were measured alongside the same osteoclast negative control cultures used in previous experiments (*see 5.4.6.1*). **Figure 75** shows significant progesterone levels were found alongside osteoclast media measurement following removal of media blanks (P=0.027 n=5). With all adipocyte cultures grown from biopsies and matured from 2-3 passage preadipocytes, we were able to control and maintain all growth conditions. Although the progesterone levels measured from both adipocyte cultures are significantly lower than those examined within the positive theca (intra) control (**Figure 75**) the osteoclast readings support these results as more than background levels. This is further supported through the media blanks and the sensitivity of the chosen ELISA (*see 3.7*). With no progesterone exposure *in vitro* these findings are particularly interesting.

5.4.6.3 Adipocyte androstenedione secretion

Based on the presence of the precursor involved in the gonadal steroidogenic pathway involved in androstenedione synthesis (*see 1.3*); and determination of the key enzyme necessary to locally synthesis androstenedione (*see 5.4.6*). We next examined androstenedione levels. The extracellular levels were measured in media taken from non-PCOS cultures (n=12) following 3-day maintenance in controlled conditions using a specific androstenedione ELISA (Genway 40-056-205044).

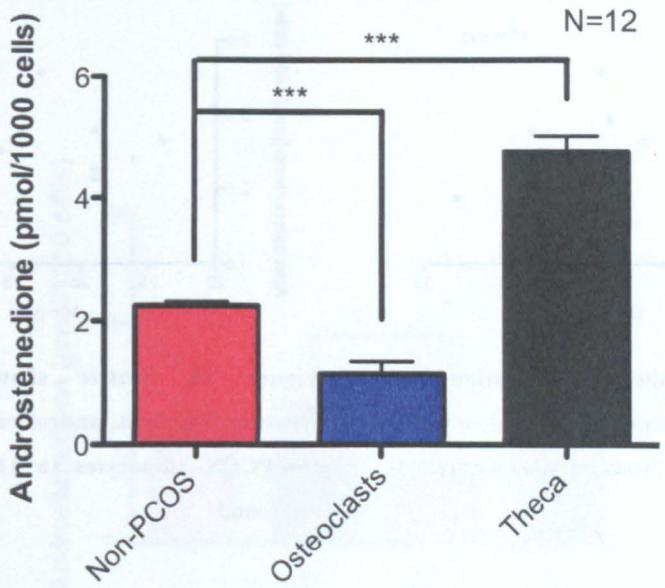

Figure 76. Non-PCOS mature adipocytes androstenedione secretion. 3 day media was harvested from non-PCOS adipocyte cultures and measured for androstenedione levels against negative control osteoclast media harvests and positive control theca media harvest. Results are mean ± SEM. Significant readings were seen against the negative control using one-way ANOVA and *bonfferoni* post hoc tests statistical examination (***P<0.001 n=12).

The presence and levels of androgen within media harvests taken from our non-PCOS mature adipocytes were measured using the ELISA technique. **Figure 76** shows androstendione was present within the media examined. These readings were supported through cultured theca cells media harvests, which have been proven to secrete high levels of androstendione *in vitro* [91]. Although androstendione levels were determined, measurements taken from theca secretion did show significantly higher levels within the media harvests (***P<0.001). Unsupported these readings could be suggestive of background levels however the ELISA kit used was chosen for its specifity and sensitivity with readings capable of examination as low as 0.019 ng/ml with adipocyte readings showing mean 0.125 ± 0.002 ng/ml. In order to further support these levels negative controls were used through osteoclast harvests and media blanks. **Figure 76** shows significant decreases were found in adipocyte against negative controls (***P<0.001).

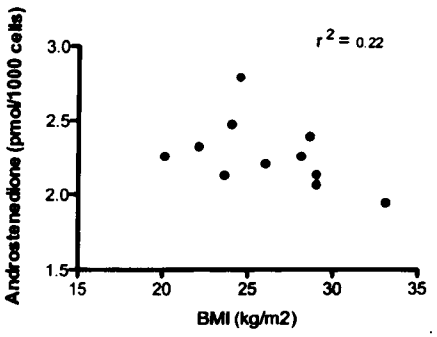

Figure 77. Scatter showing correlation between BMI and androstenedione levels non-PCOS adipocytes (n=11). No correlation was found.

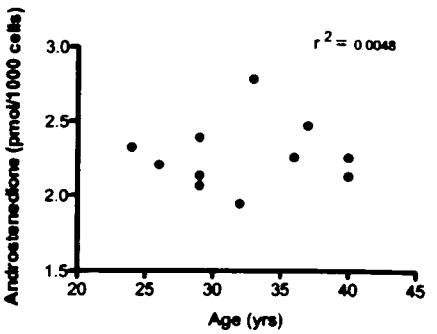

Figure 78. Scatter showing correlation between Age and androstenedione levels in non-PCOS adipocytes (n=11). No correlation was found.

The non-PCOS samples chosen for examination allowed ranges across premenopausal age (24-40) and BMI (20-33). Although sample numbers were limited this allowed a limited study of correlations that may exist between the androstenedione levels found within media harvests and these patient factors. Examination of non-PCOS samples showed no correlation existed between either age or BMI in the levels of androstenedione determined (**Figure 77**, **Figure 78**).

5.4.6.4 Comparison of non-PCOS and PCOS androstenedione secretion

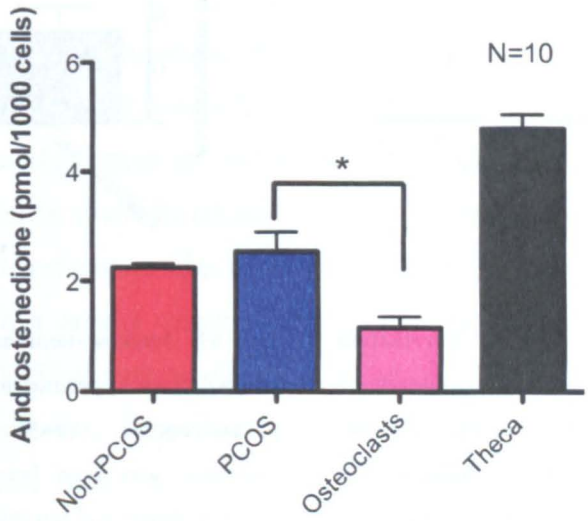

Figure 79. PCOS mature adipocytes androstenedione secretion against non-PCOS and controls. 3 day media was harvested and measured for androstenedione levels against non-PCOS secretion and negative control osteoclast media harvests and positive control theca media harvest. Results are mean ± SEM. Significant readings were seen against the negative control following one-way ANOVA and *bonfferoni* post hoc tests (*P<0.05 n=10).

PCOS adipocytes were also cultured to maturity and assayed for 3 days before media harvests were examined for androstenedione using the same protocol as non-PCOS harvest (*see 3.6*). Theca and osteoclast controls were used to support findings and the same ELISA kit was used. Results showed similar levels of androstenedione within PCOS mature adipocyte media. No significant differences were seen between non-PCOS and PCOS groups **Figure 79**, and when results were examined across patient samples for correlations in androstenedione against BMI and age no correlation was found.

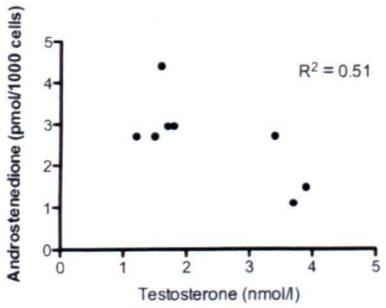

Figure 80. Scatter showing correlation between testosterone and androstenedione levels in PCOS adipocytes (n=8). Negative correlation was seen with androstenedione decreasing with increased levels of testosterone (n=10 / $R^2 = 0.51$).

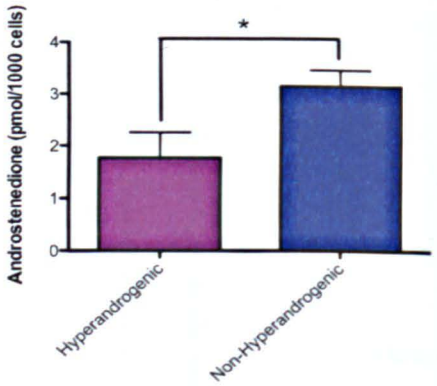

Figure 81. Androstenedione levels from media harvested from hyperandrogenic (n=3) and non-hyperandrogenic (n=5) PCOS samples. Significance was seen between androstenedione levels from hyper and non-hyper androgenic PCOS samples following unpaired *t-test* (*P<0.01). Results are mean ± SEM

Examination across hyperinsulinemic and hyper secretion of LH groups also showed no correlation existed. However examination of testosterone levels in hyperandrogenic and non-hyperandrogenic PCOS sample medium correlation was found. **Figure 80** shows that in samples taken from PCOS patients with increased testosterone levels adipocyte androstenedione levels within the media harvests would decrease. Further examination found that androstenedione levels between hyperandrogenic samples were significantly reduced than those measured in non-hyperandrogenic samples (P = 0.047) (**Figure 81**).

5.5 Discussion

5.5.1 Expression of the steroidogenic enzyme CYP17 within pre and mature adipocytes

Immunofluorescence and comparative phase contrast, showed clear cell morphology and fluorescence representative of CYP17 expression in the 3T3-L1 preadipocyte cytoplasm as well as the human pre and mature SC adipocyte cultures. Blouin *et al.*, (2009) compared human omental and subcutaneous samples for steroidogenic activity at both preadipocyte and mature adipocyte stages [281]. They found increased activity in SC cultures with a greater expression of the steroidogenic enzymes AKR1C3, RDH5, AKR1C2, P-450 aromatase, steroid sulfatase, ERα and 17β-HSD-3, following maturation. We however, found perinuculear staining for CYP17 within our preadipocyte cultures, which pointed to active protein synthesis at this stage of adipocyte development. This coupled with increased expression against our mature adipocyte cultures shown by our western blot analysis suggests an increase of this particular steroidogenic enzyme during the preadipocyte phase. Interestingly, Blouin also reports CYP19 increases following differentiation contradictory to reports by Mcternan *et al.*, (2002) and Dieudonne *et al.*, (2006) who both report CYP19 mRNA reduction in mature / differentiated SC cultures [276, 282]. Decreased CYP19 expression following differentiation may support our findings. If an androgen synthesising pathway exists within adipocytes, increased oestrogen synthesis may require greater CYP17 expression to satisfy the necessary precursor. Although Blouin's findings show the opposite results, he accounts for variation in findings as a result of discrepancies in RNA isolation in the presence of large amounts of lipids [276]. By using our protocols to examine aromatase expression we would be able to examine these reports further and look into the possible existence of this pathway.

Although our mature adipocytes showed fluorescence surrounding the lipid droplets, the technique proved inadequate in pinpointing the exact localization of CYP17. This may be as a result of the discrepancy in z-plane phase. This may also be linked to cellular debris that tends to accumulate during differentiation. We also found fluorescence occurring in some larger lipid droplets. This again may be as a result in phase variation or debris and could be further explored utilizing the 3 dimensional

images acquired through confocal microscopy. It may be that CYP17 is expressed within lipid droplets. This may also be as a result inadequate fixation leading to rupture of some lipid droplets and subsequent protein seepage into this area of the cell. Although acetone-methanol fixation is recommended for cytoplasmic based proteins it is particularly harsh and can damage the fragility of lipid droplets. This rupture would therefore explain the discrepancy seen where cells have retained cytoplasmic fluorescence surrounding the lipid droplets. For this reason paraformaldehyde fixation may be more suitable when examining the human primary cultures as recommended by Mallide, 2008.

By showing the existence of the steroidogenic enzyme CYP17 and its cellular localization within both preadipocytes and mature adipocytes we have supported the validity of using these protocols and primary cultures as well as our animal cultures to examine steroidogenesis within adipocytes. This in turn has also given evidence towards the possible capability of adipocytes to locally synthesize androgens. Currently adipocytes are known to take part in steroidogenic activity with their role in oestrogen and androgen synthesis [127, 283, 284] however to date this is believed to utilize circulatory precursors. Examination into this area would therefore assist the current understanding of adipocyte metabolism.

Both the immunofluorescence and western blot results also show that CYP17 is present prior to and following differentiation. Studies into the effects of oestrogen on preadipocytes have shown a positive influence on proliferation [285] and with the importance of androgen as a precursor for oestrogen synthesis the presence of CYP17 and subsequent androgen synthesis, may be linked to proliferation. No variation in expression across either our non-PCOS or PCOS samples was seen.

Examination of our non-PCOS against PCOS human adipocyte cultures looking at CYP17 expression, did show slight discrepancy within our control results. CHO were used as a positive comparison for CYP17 and the band exposure was as strong as those in both non-PCOS and PCOS samples. Based on the other results and knowing that the CHO was used as an intra control we would expect the intensity to be stronger. This may be explained through repeated use of control lysate, which were used within as many experiments as possible to allow for continuity. However over time these lysates would degrade from freeze thawing or on some occasions run out. When this

occurred new CHO cultures would be grown to replace. Where possible the replacement lysates would be run on a known samples repeating a past blot. The results seen here may be a CHO lysate beginning to degrade. They may also be the result of variation in a new CHO cell line due to a further passage and subsequent generation change. Time constraints meant we could not repeat the experiment. For the purpose of showing CYP17 presence between groups this result was sufficient but maybe considered in future work.

The existence of androgen receptors (AR) in both pre and mature adipocytes further supports the presence of an androgen pathway. It has also been shown that ARs are more abundant within preadipocytes more so in human/animal model comparison [228], which support a greater involvement of androgen at the preadipocyte stage of development. This is suggested by our finding of significantly greater levels of CYP17 expression within our 3T3-L1 and human preadipocytes.

The importance of androgens on the inhibition of adipocyte proliferation has been shown in animal models along with its ability to inhibit differentiation [285-287]. By showing the existence of CYP17 both pre/post differentiation, we find suggestion that androgen may also play an important regulatory role and therefore require localized synthesis. We therefore continued examination of these findings using human cells as to develop the study further.

5.5.2 Hormone secretion in non-PCOS and PCOS adipocytes

Both our findings and the reported existence of other steroidogenic enzymes in human adipocytes points to the validity of localised hormone secretion (*see 1.13*). With androstenediones relationship to PCOS and the association PCOS has to BMI we wanted to examine this theory further in adipocytes.

Progesterone plays a vital role as a precursor within ovarian androstenedione synthesis. By determining its presence we could further support the requisites necessary for a steroidogenic pathway. Careful control of the culture environment ensured limited exposure to supplements that may have confounded results and the inclusion of a negative and positive control along with sufficiently sensitive techniques, meant that the progesterone presence/levels found could be supported. Progesterone was seen in both non-PCOS and PCOS media harvests and shown to be

significant against the negative controls. Although no significant variation was seen between the two groups, a trend did seem to suggest increased progesterone levels within the PCOS samples that may require further analysis through increasing the sample size. It is understood that progesterone is synthesised by the ovary, testis adrenal glands and the placenta during pregnancy. Although it is yet to be established, it is also believed that neurons also have the ability to locally produce progesterone. If this were true it maybe produced via P450scc/desmolase action on the cholesterol precursor and converted to pregnenolone for further conversion by dehydrogenase (*see 1.3.2*)[46, 47]. With adipocytes shown to have the necessary proteins and related precursors necessary to also locally biosynthesise progesterone the possibility remains feasible and supports our findings of progesterone presence in isolated adipocyte cultures. However, progesterone can also be stored at any stage of preadipocyte development, as can any of the precursors required for synthesis. It may be possible to prove this theory through increasing the passage of the cells examined. The stores may then be utilised through increased passage of the preadipocyte cultures and the lack of supplements required to replenish the stocks. This would however reduce physiological comparison

With the discovery of the progesterone receptor by O'Brien (1978) more evidence pointed to progesterone's involvement in adipocyte metabolism. Secretion from localised production may allow self-regulation and research has shown that progesterone may be involved in the regulation of lipogenesis through stimulation of transcription factors such as ADD1/SREBP1c [288]. Progesterone is also known to be stored within adipocytes and used for progressive release [289]. Although this would explain its presence within our harvests, our samples were 3rd generation primary cultures differentiated from preadipocytes and therefore we would expect any cholesterol stores to be exhausted through passage.

Other steroid hormones have been shown to influence adipocyte proliferation including androgens and oestrogens [283]. Based on these reports progesterone's involvement in adipocyte metabolism is not entirely understood.

Repeating the ELISA on the same media harvest also showed the presence of androstenedione in both non-PCOS and PCOS samples. Similar to the progesterone results, no significant variation across groups was seen.

With reported evidence supporting the involvement of androstendione in adipocyte metabolism a localised production and subsequent secretion seems founded and has been previously theorised [281]. However its involvement in adipocyte metabolism is not fully understood and validation of this biosynthesis is required as well as mapping of the pathway involved. In doing so we would get some idea of not only the effect but also the influences this may have on the physiological condition. Furthermore we could examine any associations this may have in endocrinolgical disorders. Our study attempted to go someway to introduce this with focus on PCOS. Although limited by our samples size we began a pilot correlation of androstendione secretion across BMI, age and symptomatic hyperandrogenimic and hyperinsulineamic patients in non-PCOS and PCOS samples. Our non-PCOS samples showed no correlation in BMI or age with regard to androstenedione secretion. Similarly in the PCOS group no correlation was found in BMI, age hyperinsulineamic or hypersecretion of LH. However our PCOS group interestingly did show a negative correlation between testosterone levels and androstenedione secretion. This may suggest that a reduction in androstenedione is as a result of increased demand/conversion to testosterone. This result therefore justifies further examination.

Limited availability of PCOS biopsies meant that we could not control for all factors that may affect the levels of androstenedione secretion. Our results were based in accordance to BMI, hyperandrogeneamia / hyperinsulinemia groups. We have suggested that a negative correlation may exist between hyperandrogenic samples and androstenedione secretion showing a decrease in androstenedione with increased testosterone. If this were true it would explain a possible associated to the lack of significance in androstenedione seen between our non-PCOS and PCOS groups knowing that testosterone is typically elevated in PCOS samples.

6.0 HORMONAL INFLUENCES ON ADIPOCYTE FUNCTION IN NON-PCOS AND PCOS WOMEN

6.1 Introduction

Approximately 50% of women with PCOS are overweight or obese [173]. Obesity is believed to pose possible involvement in the development of PCOS with abdominal obesity showing links to insulin resistance and the associated hyperinsulinemia asymptomatic of PCOS [173, 278]. Further research has shown that obesity related hyperandrogenemia found in PCOS may play a key role in the pathogenesis of hyperinsulinemia [290]. The increased metabolic dysfunction shown in both non-PCOS and PCOS obese patients also offers mechanisms for associated conditions; increased oestrogen production rate [173], increased activity of the opioid system and of the hypothalamic-pituitary-adrenal axis [291, 292], decreased sex hormone binding globulin synthesis [293] and, possibly, high dietary lipid intake [173, 294]. Whether these links are valid or not, enough evidence is available to suggest increased severity of hyperandrogenism and related clinical features seen in obese PCOS women [115, 278]. Patients with PCOS are often encouraged to reduce their weight, which has been shown to be beneficial to improving clinical and endocrinological features [118, 295]. The addition of insulin sensitizing agents and anti-androgens to weight loss programs, have also been seen to give positive results, reducing hisuitism, endocrinoligical abnormalities and amenorrhea [296, 297]. This further supports the possibility that insulin related dysfunction associated to PCOS may correlate to a possible androgen adipocyte relation, and studies have shown that androgen significantly decreases levels of differentiation in both omental and subcutaneous adipocyte cultures this may in turn lead to a direct effect through increased proliferation in preadipocytes [209, 298] or an increase in the availability of substrate necessary to increase oestrogen synthesis which has been shown to encourage adipogenesis in mature adipocytes [276].

Luteinising hormone is a key endocrine factor in PCOS both in its positive effect on steroidogenic responses seen in ovarian androgen secretion [299] and its symptomatic hyper secretion [145]. Little is known about luteinising hormone's action on

adipocytes with only recent studies into the effects of human chorionic gonadotropin (HCG) on adipose development, suggesting the existence of a luteinising hormone receptor (LHR) [300]. With the increased association of LH secretion with PCOS [145, 301], further investigation is of clinical relevance and it is currently unknown if PCOS abnormalities in LH hyper secretion are primarily due to sensitivity to GnRH, or secondary to the influence of sex steroids [145, 302]. Furthermore, both *in-vitro* and *in-vivo* evidence exists as to the possible synergistic effects of insulin and LH on ovarian metabolism with Cara and Rosenfiled (1988) showing controlled increases in LH and IGF-1/insulin correlating to increased androgen secretion within rat theca cells [302-304]. It is understood that adipokines, secretory products of adipocytes, act to communicate with the brain, however it has only been in the last 6 years that the discovery of pituitary hormone and hypothalamic peptide receptors including ACTH, TSH, GH, prolactin, and vasopressin within adipocytes has led to the suggestion that the brain may any influence metabolic function [305]. Mapping of adipocyte structure and function has allowed adipocytes to no longer be regarded as an inert tissue with its importance as an endocrine organ under the possible influence of hypothalamic-pituitary-adipose axis requiring investigation due to its possible clinical impact. With these realisations and with the relationship adipose plays with many physiological conditions, further examination into the role adipose may play as a possible therapeutic target is of great importance. Biochemical dysfunction is central to PCOS and the possible involvement adipocytes may therefore play, was a focal point in my study.

6.2 Objectives

Our first objective was to ensure that an LH receptor existed within adipocytes and in doing so we would allow evidence of an LH/adipocyte relationship. Once this was achieved we would examine whether receptor expression remained sufficient under biochemical levels found in PCOS (LH and insulin). This would justify further examination into the effects these biochemical levels would have on various aspects of adipocyte metabolism.

i. Determine the presence of the LH receptor within pre/mature human adipocytes and therefore determine a possible relationship between LH and adipocyte metabolism.
ii. Determine whether altering insulin and LH concentrations *in-vitro* influence LH receptor expression and therefore determine whether the biochemical conditions found in PCOS allow adipocyte/LH relationship.

On determination of adipocyte LHR expression under PCOS biochemical conditions, we examined three areas associated to increased androgen contribution under the same condtions. This focus was taken from our comparative ovarian theca study and theories associated to PCOS ovarian hyperandrogenemia (Munir *et al.*, 2004)

iii. Determine whether altering insulin and LH treatments influences non-PCOS and PCOS adipocyte proliferation.
iv. Determine whether altering insulin and LH treatments influences steroidogenic enzyme expression in non-PCOS and PCOS adipocytes.
v. Determine whether altering insulin and LH treatments influences progesterone and androstenedione secretion by non-PCOS and PCOS adipocytes

6.3 Results

6.3.1 Expression of LH receptor in non-PCOS and PCOS adipocytes

Non-PCOS and PCOS adipocytes were cultured from human biopsies (n=6) and examined for LH receptor using the immunofluorescence technique (*see 3.5.1*). Primary preadipocyte cultures were cultured until 40% confluent to allow examination of individual cell morphology as was seen in phase contrast images (**Figure 83**, **Figure 85**). Positive and negative controls were used to support all findings and included preadipocytes markers, leptin immunoreactivity (anti-human OB R&D Systems (AF398)) commercially available cell line preadipocyte / mature adipocyte comparisons (PromoCell C-12730), and β-actin. Negative controls included human leukocyte cells for marker comparisons and secondary antibodies alone.

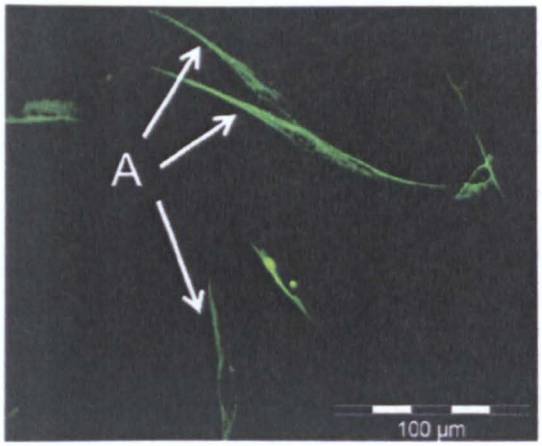

Figure 82. Human preadipocyte expression of LHR. Human non-PCOS preadipocytes stained with LHR and FITC conjugated anti-rabbit secondary antibody. **(A)** Fluorescence is localised to the cell membrane while nuclei are un-stained. This suggests LHR is located within the cells membrane.

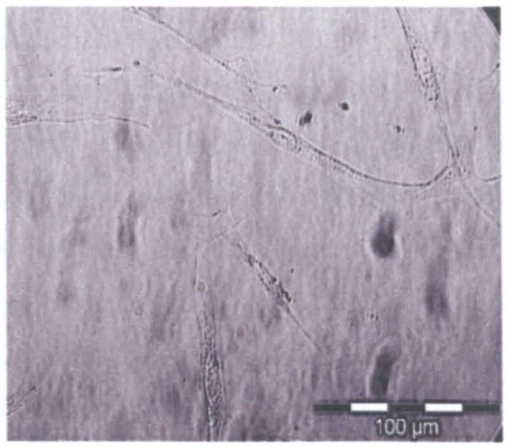

Figure 83. Matched phase contrast micrograph of human preadipocytes. Human non-PCOS preadipocytes grown to 40% confluence allowing determination of cell morphology to be seen in both phase contrast image and on comparison to incubation with fluorescent antibody probe (**Figure 82**)

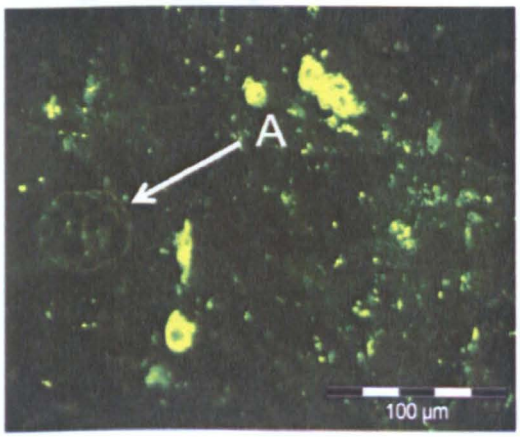

Figure 84. Human mature adipocytes expression of LHR. Mature adipocytes stained with LHR and FITC conjugated anti-rabbit secondary antibody. Fluorescence is weak and varied due to cell debris and lipid formation however fluorescence suggests LHR exists within the cells membrane of the adipocyte.

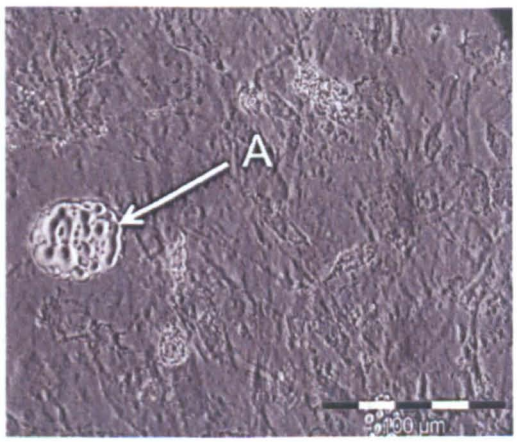

Figure 85. Matched phase contrast micrograph of human mature adipocytes. Adipocytes grown to 90% confluence allowing maturation. **(A)** shows fully matured adipocyte identified through lipid droplet formation.

Figure 82 shows localised fluorescence around the preadipocyte membrane. Fluorescence can also been seen within differentiated adipocytes as seen in **Figure 84** however this was observed to be lower levels of fluorescence within the mature

adipocyte cultures, suggestive of reduced expression. This finding was consistent throughout experiments (n=6). To further examine this, both preadipocyte and mature adipocyte lysates were harvested from human primary cultures (n=4) and LHR protein expression was assayed using western blotting. **Figure 86** shows a western blot for LHR expression using the polyclonal LHR antibody (Santa Cruz SC25828). An immunoreactive band at the predicted size of 85kDa was detected. Lane 1 gives expression of LHR within non-PCOS preadipocyte lysate. β-actin was used to control for protein loading and following this, comparison to mature adipocyte LHR expression shown in lane 2, showed significant reduction suggestive of reduced LHR expression. Shown expression of LHR at either stage of adipocyte development is however indicative of a possible LH-adipocyte relationship and further encourages investigation of potential autocrine metabolic functions within adipocytes.

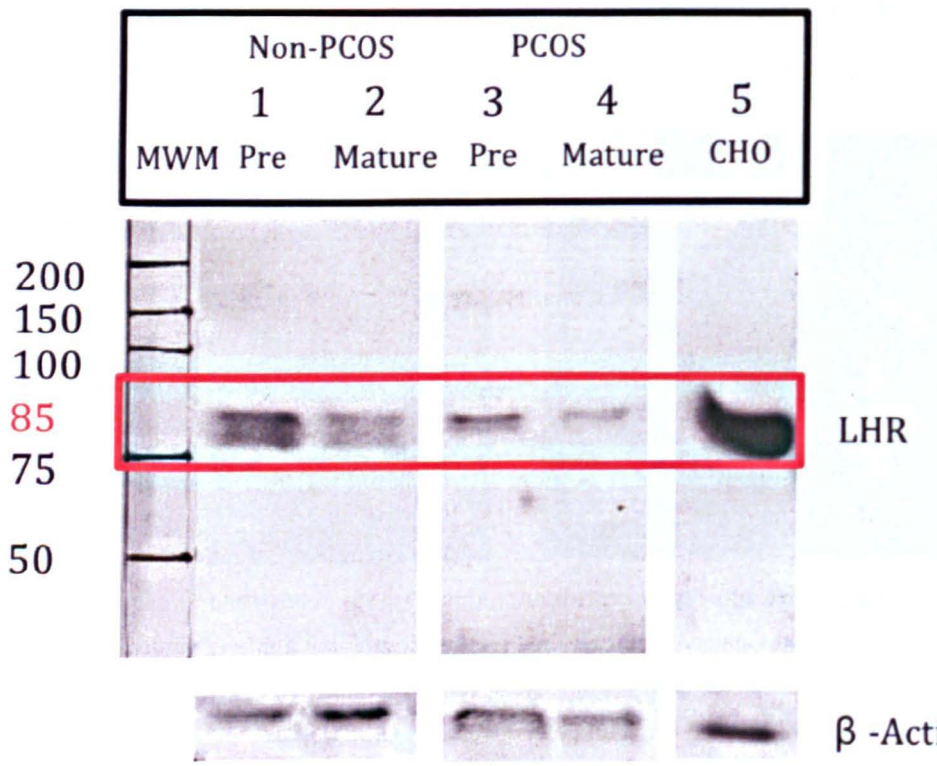

Figure 86. Western blot LHR expression in non-PCOS pre and mature adipocyte (lane 1 and 2) against PCOS pre and mature adipocytes (lane 3 and 4). Pre and mature adipocyte lysates from non-PCOS and PCOS cultures (n=4) were probed using LHR (H-50) antibody. Exposure at 640sec shows bands at 85kDa, which were predominant in preadipocyte samples of both non-PCOS and PCOS lysates. The blot was then stripped and re-probed using β-Actin to control for protein loading. Abundant LHR expression is evident in CHO lysate, which was used as a positive control.

These experiments were continued using human PCOS preadipocyte and mature adipocyte cultures to examine possible variations in expression of LHR (lane 3 and 4) however based on the results obtained from the western blotting (**Figure 86**) no significant difference ($P>0.05$) based on band intensity was found, although observation of the western blot (**Figure 86**) showed a predominant double banding effect within the non-PCOS lysates. These results did however further support the variation seen in the non-PCOS group with reduced band expression in mature PCOS samples indicative of reduced expression of LHR in differentiated cultures.

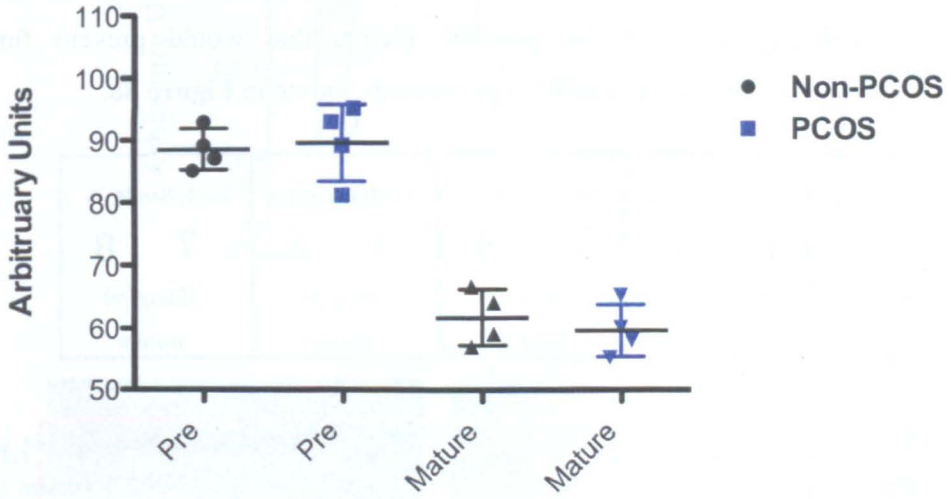

Figure 87. Summarising the densitometry representative of average expression of LHR in preadipocyte and mature human samples from both non-PCOS and PCOS. Using β-Actin normalisation to compare levels of band intensity in western blot LHR expression Densitometry examination of western blot results using unpaired *t-tests* (**Figure 86**) showed no significant difference was seen in expression of LHR between preadipocytes of non-PCOS and PCOS groups (n=3 *vs.* n=3). This was also true for comparison of non-PCOS and PCOS mature adipocyte lysate (n=3 *vs.* n=3).

6.3.1.1 The effects of insulin and LH treatment on LH receptor in non-PCOS and PCOS adipocytes

The results showing the expression of the LH receptor in primary cultures from both non-PCOS and PCOS biopsies allowed us to further examine hormonal influences on adipocytes typically associated with the PCOS condition. These include hyperinsulineamia and hypersecretion of LH. The effects of varied hormonal treatments on the expression of LHR were initially investigated. It was necessary to study the effects of these conditions on LHR expression before further examination of subsequent adipocyte metabolism, as these results may lead to an association between conditions and expression, and/or possible factors that would prevent further examination such as inhibition of LHR expression as shown in **Figure 88**.

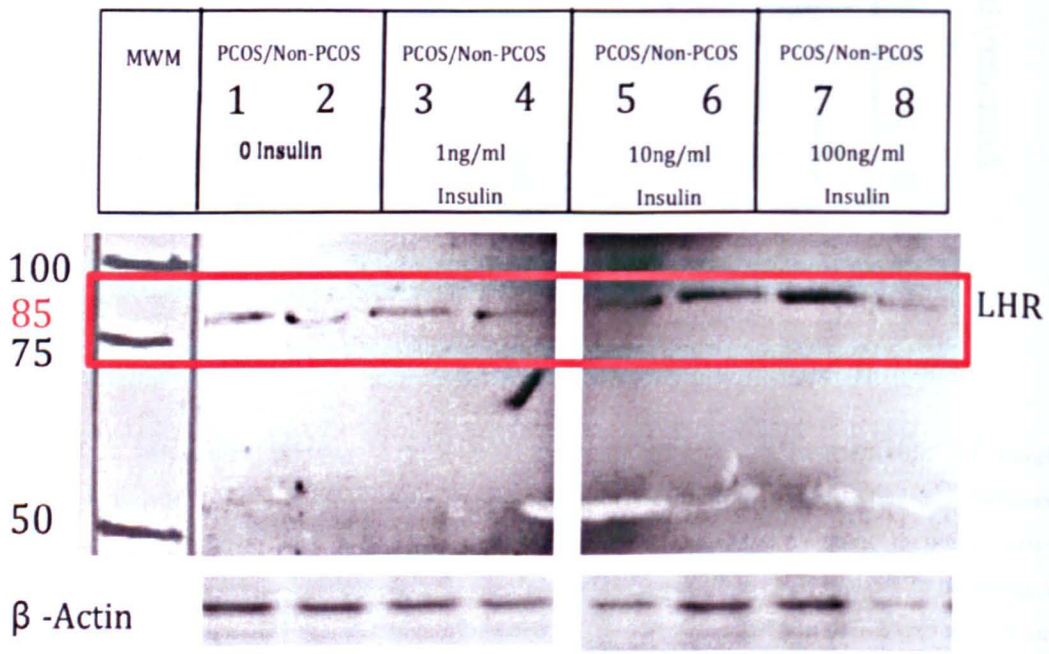

Figure 88. Western blot LHR expression in PCOS (lanes 1,3,5 and 7) against non-PCOS mature adipocyte (lane 2,4,6 and 8) under varied insulin (n=3 *vs.* n=3). Mature adipocyte were treated with varying doses of insulin *in-vitro* (insulin 0 (lane 1-2) 1ng/ml (lane 3-4) 10ng/ml (lane 5-6) 100ng/ml (lane 7-8). Cells were then harvested and prepared for WB. Lanes were probed using LHR (H-50) antibody. Exposure at 580 sec shows immunoreactive band at 85kDa with increased LHR protein correlating with increased insulin treatment. Little variation was seen between non-PCOS and PCOS samples. The blot was then stripped and re probed using β-Actin to control for protein loading.

Our initial experiments focused on insulin treatment alone in non-PCOS and PCOS samples. Western blotting was used to analyse LHR expression in PCOS (lanes 1,3,5 and 7) and non-PCOS lysates (lane 2,4,6 and 8) following varied insulin treatments (0-100 ng/ml). The experiments were focused on mature adipocyte cultures (n=3) due to their reported increased steroidogenic enzyme expression and activity [228, 282].

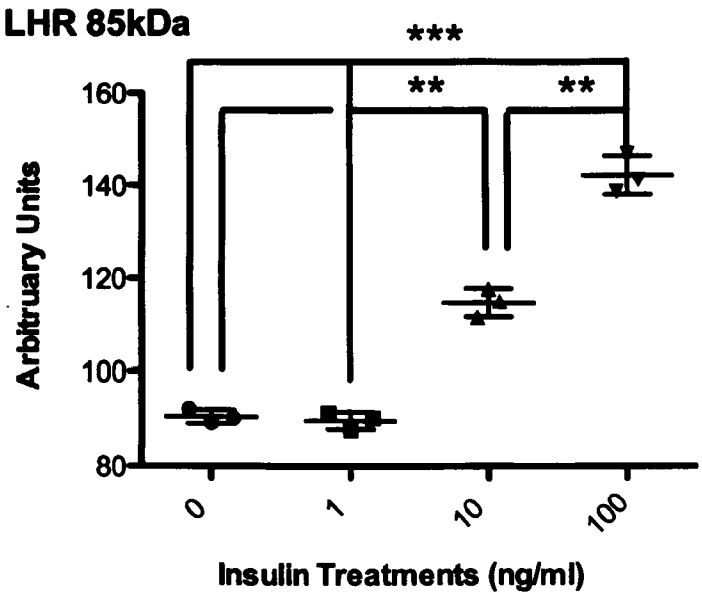

Figure 89. Summarising the densitometry representative of average expression of LHR in non-PCOS mature adipocytes (n=3) under varying insulin treatments. Using β-Actin normalisation to compare levels of band intensity in western blot LHR expression **Figure 88** densitometry graphs were created (as shown here). Significant differences were seen in expression of LHR with increases in insulin treatment of 0-10 and 10-100 ng/ml and examined using one-way ANOVA. Data shows mean / ± SEM. Significance was seen from 0ng/ml-10ng/ml (**P<0.01) and 0-100 ng/ml (***P<0.001). Significance was also seen from 1-10ng/m (**P<0.01) and 1-100ng/ml (***P<0.001). Finally significant increases in LHR expression were seen between treatments of 10-100ng/ml (**P<0.01).

Analysis of LHR expression showed increased LHR protein in response to increased insulin treatment with a sudden increase seen from 1-10ng/ml in progressive treatments (lane 3-4 (1 ng/ml) to lane 5-6 (10 ng/ml)). Levels of band intensity were quantified and compared against β-Actin to give statistical analysis of LHR expression. No significant variation was seen between within treatment groups across non-PCOS and PCOS lysates. **Figure 89** and **Figure 90** show significant increases between insulin treatments following 1ng/ml 1-10 ng/ml (**P<0.01), 1-100ng/ml (***P<0.001) and 10-100ng/ml (**P<0.01) in both non-PCOS and PCOS groups. A

large increase in LHR band intensity (**Figure 88**) and expression (**Figure 89**) was seen between 0-10ng/ml, suggesting 10 ng/ml was optimal level required to cause increase LHR protein above basal. PCOS samples showed similar levels of LHR protein. In conclusion these results suggest no significant variation in LHR expression is seen between non-PCOS and PCOS mature adipocyte within insulin treatment groups.

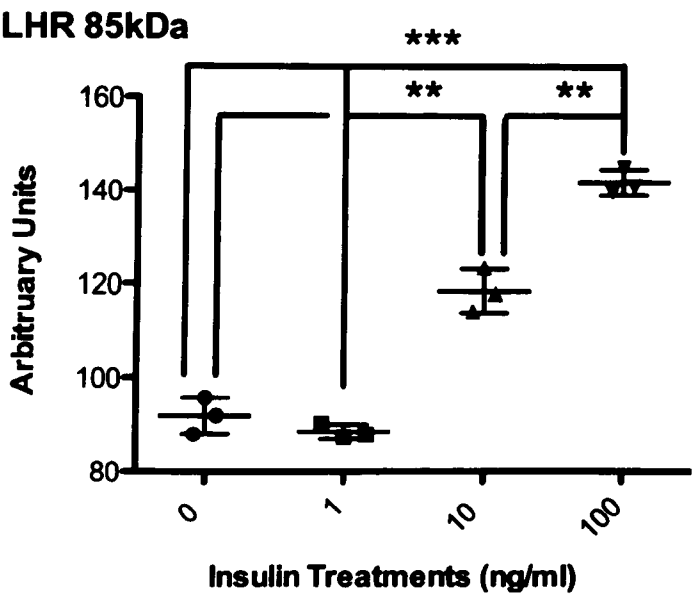

Figure 90. Summarising the densitometry representative of average expression of LHR in PCOS mature adipocytes (n=3) under varying insulin treatments. Using β-Actin normalisation to compare levels of band intensity in western blot LHR expression (**Figure 88**) Densitometry examination of western blot results (as shown here). Significant difference were seen in expression of LHR with increases in insulin treatment of 0-10 and 10-100 ng/ml using one-way ANOVA. Data showed examination of mean / ± SEM. Significance was seen from 0ng/ml-10ng/ml (**P<0.01) and 0-100 ng/ml (***P<0.001). Significance was also seen from 1-10ng/m (**P<0.01) and 1-100ng/ml (***P<0.001). Finally significant increases in LHR expression were seen between treatments of 10-100ng/ml (**P<0.001).

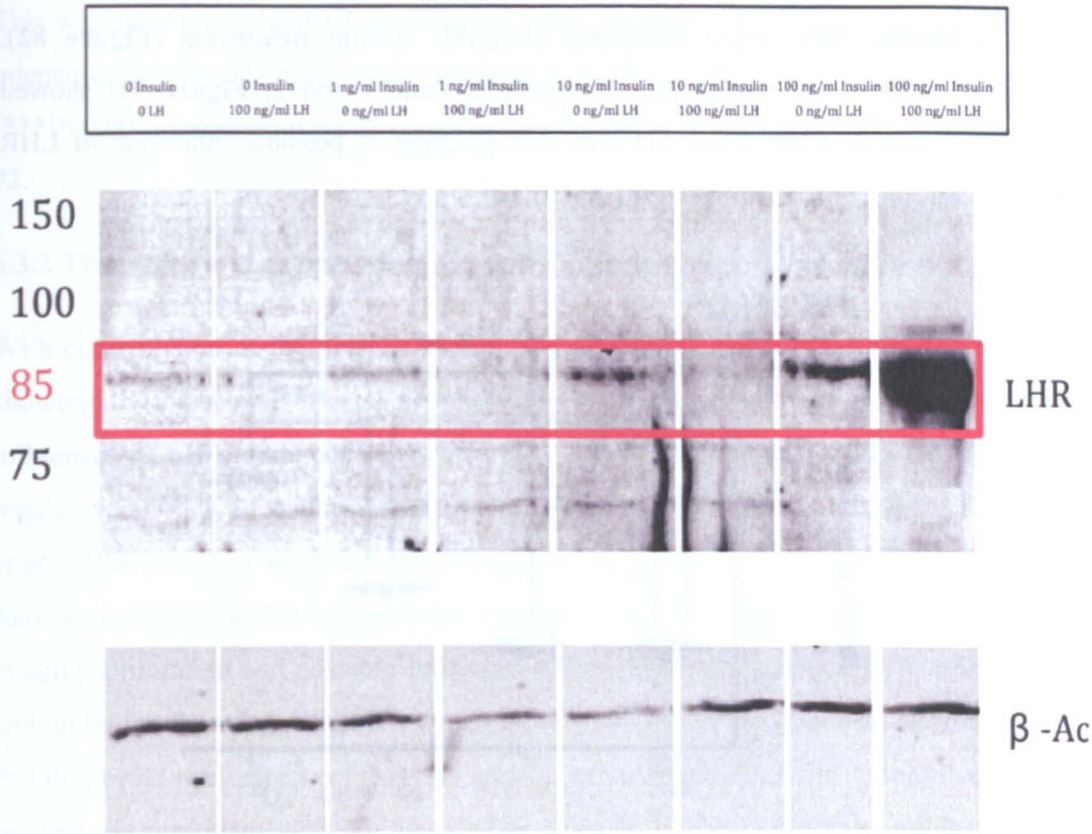

Figure 91. Western blot LHR expression in non-PCOS mature adipocyte (lane 1-8) under varied insulin and LH treatments combined (n=4). Mature adipocyte were treated with varied doses of insulin alone or in combination with LH *in-vitro* (insulin 0 (lane 1-2) 1ng/ml (lane 3-4) 10ng/ml (lane 5-6) 100ng/ml (lane 7-8) / with LH 100ng/ml (lanes 2,4,6 and 8). lanes were probed using LHR (H-50) antibody. Exposure at 580sec shows increasing band exposure at 85kDa in lanes 1,3,5,7 and 8. The blot was then stripped and re-probed using β-Actin to control for protein loading.

We expanded our study to examine LH treatment alone and in combination with the insulin range used previously (*as discussed in 6.1*). Western blot experiments were carried out on non-PCOS lysates (n=4) to examine both insulin and LH treatments combined. Exposure time was reduced to 580secs due to overexposed band seen in lane 8 **Figure 91** under treatment levels of 100 ng/ml insulin and LH. All results were examined alongside CHO cell line positive controls shown to express LHR [306]. LH treatments of 100 ng/ml, based on the ranges chosen in previous studies for optimal responses [91, 144] were used to examine the effects on LHR protein levels in both non-PCOS pre and mature adipocytes. Insulin treatments were also examined with ranges including 0,1,10 and 100ng/ml. Decreased LHR protein levels were seen after

LH treatments alone when compared alongside insulin treatments (**Figure 82**). Comparison of 0 and 100ng/ml LH treatment (lane 1 and 2, **Figure 91**) showed reduced levels of LHR when LH was zero pointing to possible inhibition of LHR expression.

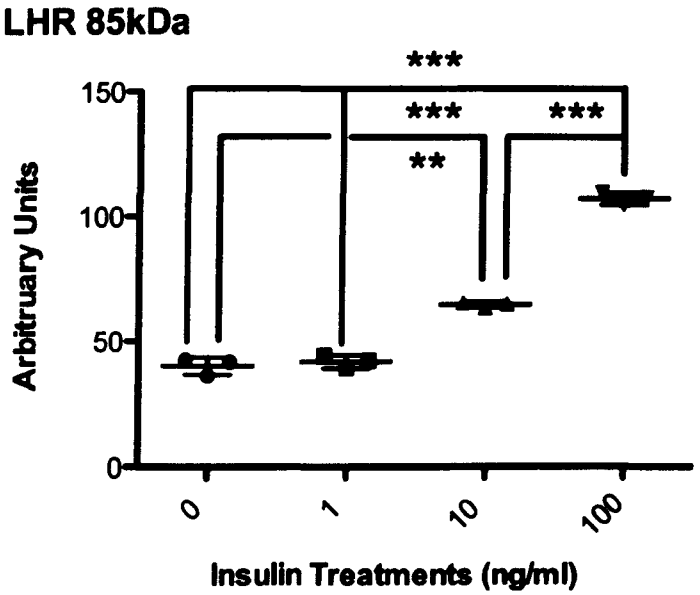

Figure 92. Summarising the densitometry representative of average expression of LHR in non-PCOS mature adipocytes (n=4) under varying insulin treatments. Using β-Actin normalisation to compare levels of band intensity in western blot LHR expression (**Figure 91**) Densitometry examination of western blot results (as shown here) showed significant difference were seen in expression of LHR with increases in insulin treatment of 0-10 and 10-100 ng/ml Data showed examination of mean / ± SEM using one-way ANOVA. Significance was seen from 0ng/ml-10ng/ml (***P<0.001) and 0-100 ng/ml (***P<0.001). Significance was also seen from 1-10ng/m (**P<0.01) and 1-100ng/ml (***P<0.001). Finally significant increases in LHR expression were seen between treatments of 10-100ng/ml (***P<0.001).

Increased insulin treatments showed a positive relationship with LHR protein levels under the treatment range 0–10ng/ml insulin (lanes 3, 5 and 7, **Figure 91**). Similar levels of LHR protein were seen at both 10 and 100 ng/ml insulin treatments (lanes 5 and 7) as was seen in **Figure 86**. However, reduced LHR expression was seen with LH treatment and shown within insulin/LH augmentation (4 and 6) with LHR protein at 1-10ng/ml insulin decreased with the addition of 100ng/ml LH (**Figure 91**). Interestingly, the combination of 100 ng/ml insulin and 100 ng/ml LH showed the highest levels of LHR protein when compared to 100 ng/ml insulin alone (**Figure 91**).

This was seen throughout all experiments (n=4). Quantification of western blot band intensities confirmed these results showing significant increases in LHR expression (***P<0.001) throughout all increases in insulin treatments alone, as shown in **Figure 92.**

6.3.2 The effects of LH on non-PCOS and PCOS adipocyte proliferation.

With confirmation of the presence of the LH receptor in adipocytes seen and results showing the hormonal effects on expression, we could begin to examine possible influences of LH on adipocyte cell responses. HCG has been shown to have varied regulatory actions dependent on target tissues both inhibitory as shown by Czerwiec *et al.*, (1989) in their work in human Leydig tumour cells; or stimulatory as seen by Rao *et al.*, (2004) within corpus lutea with research also describing LH involvement in cell proliferation and possible activation of cell death [307]. Based on these reports and under the knowledge that LH hyper secretion is typical for patients suffering from PCOS [145], the effects of LH on adipocyte proliferation is of interest. This is particularly important with the association adipocytes and obesity has within PCOS, as a dysfunction in adipocyte metabolism may be linked to the condition. Knowledge of the general effects of LH on adipocyte proliferation/metabolism would allow for comparison and possible determination of the existence of any dysfunction. We therefore began by examining the effect that LH treatment may have on preadipocyte proliferation. This would allow optimisation of treatment levels, ensure stability of our cultures under these treatments and determine possible levels of toxicity.

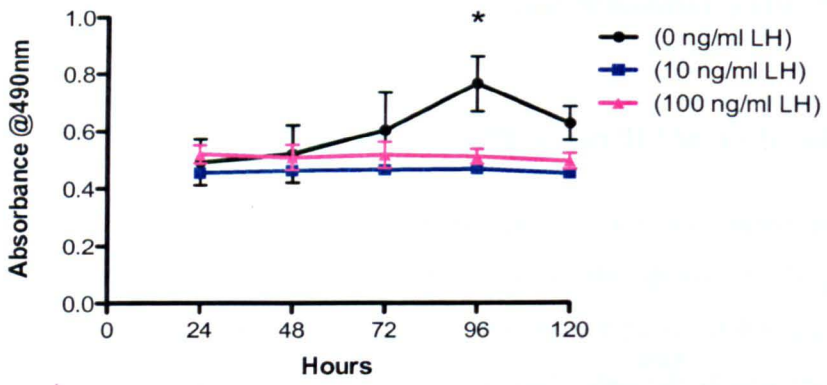

Figure 93. Non-PCOS preadipocyte proliferation under varied LH treatments. Non-PCOS preadipocyte cultures (n=5) were grown with and without varied LH treatments (10/100 ng/ml). Proliferation was measured (Abs @490) over 5 days using MTS assays. A significant decrease was seen in proliferation when examined using *unpaired t-test* between 10ng/ml treated non-PCOS on day 4 and un-treated (*P<0.046) Significant decrease was also seen on day 4 under 100 ng/ml (*P<0.032). Results are mean ± SEM.

LH treatments of 0/10 and 100ng/ml were chosen to mimic LH hyper secretion and examined in both non-PCOS and PCOS primary cultures (n=5) using the MTS assay (*see 3.4*). Non-treatment groups were used as controls and media alone also examined. All groups were found to remain stable over the five-day proliferation course with no signs of cell death seen through cellular debris or cell counts.

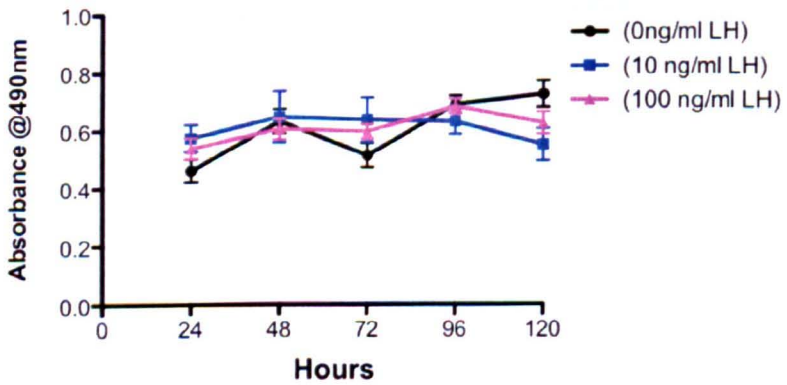

Figure 94. PCOS preadipocyte proliferation under varied LH treatments (n=5). Non-PCOS preadipocyte cultures (n=5) were grown with and without varied LH treatments (10/100 ng/ml) and proliferation measured over 5 days using MTS assays. No variation in proliferation was seen with increased LH treatment throughout cultures. Results are mean ± SEM.

Examination of non-PCOS untreated cells showed a growth curve reaching a proliferative peak by day 4 and reducing to a lower cell number at day 5 capable of being sustained by the cultures environment (**Figure 93**). Both 10 and 100ng/ml LH treatments on non-PCOS adipocytes showed consistent inhibitory effects with no significant increase in proliferation over the course of 5 days. There was a significant difference between treated and controls by day 4 (10ng/ml P=0.046, 100ng/ml P=0.032). These results suggested that the LH treatment range chosen was capable of maintaining the preadipocyte cultures with no sign of cell death however proliferation was inhibited and seen at treatments from 10 to 100ng/ml.

The proliferation assay was repeated using PCOS primary adipocytes (n=5) and the same treatment ranges. Results varied from non-PCOS with all treatments having no significant variation on proliferation when compared to non-treatment culture proliferation **Figure 94**. This suggested that the PCOS adipocyte differed from non-PCOS through lack of LH inhibition on proliferation. Closer inspection also showed that the growth pattern seen in non-PCOS proliferation was different to that seen in PCOS and although both showed similar peak proliferation in untreated groups, PCOS cells maintained these levels over 5 days whereas non-PCOS cells showed a decrease on day 5. These results gave interesting findings and points to a possible variation between non-PCOS and PCOS adipocytes they also allowed for necessary information for maintain our primary cultures and enabling us to proceed with examining hormonal effects further.

6.3.3 The effects of Insulin on non-PCOS and PCOS adipocyte proliferation

Due to its association with PCOS we also examined the effects of insulin treatments both individually and in combination with LH treatments. It would therefore be necessary to examine the effects on proliferation within our primary cultures through MTS proliferation assay.

Both animal and human studies have revealed the proliferative effects of insulin action in subcutaneous adipocytes and more so in omental adipocyte cells *in-vitro* [308, 309]. It has also been shown that increased levels of insulin treatments can be cytotoxic (400nM) to cultured human adipocytes [310]. For this reason examination of treatment range was necessary to optimize both culture maintenance and further

work. This would also allow us to determine whether variations between non-PCOS and PCOS preadipocyte proliferation under these conditions existed and if any metabolic variation may be associated.

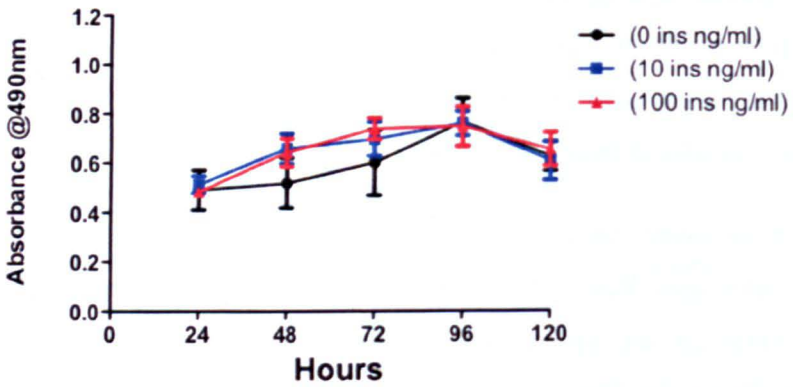

Figure 95. Non-PCOS preadipocyte proliferation under varied insulin treatments. Non-PCOS preadipocyte cultures (n=4) were grown with and without varied insulin treatments (10/100 ng/ml) and proliferation measured over 5 days using MTS assays. No significant variation in proliferation was seen under varying doses of insulin. Results are mean ± SEM.

Figure 95 shows no significant changes in proliferation occurred in non-PCOS preadipocytes. This did not vary across our treatment range (0/10/100ng/ml Insulin) [310]. These results also showed no cytotoxic effect on the primary cultures over the 5 day assay period. MTS proliferation assays were run on PCOS cultures alongside non-PCOS experiments (n=5).

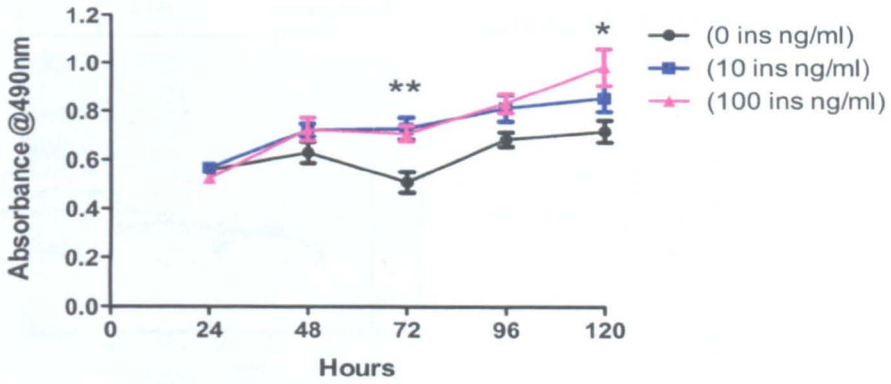

Figure 96. PCOS preadipocyte proliferation under varied Insulin treatments. PCOS preadipocyte cultures (n=5) were grown with and without varied insulin treatments (10/100 ng/ml) and proliferation measured over 5 days using MTS assays. Significant increases in proliferation on day 3 in both 10 and 100ng/ml insulin treatments against untreated was seen (**P<0.01 10ng/ml and **P<0.01 100ng/ml) with significance also seen on day 5 under 100ng/ml treatments (*P<0.05). Statistical analysis was done using unpaired *t-tests*. Results are mean ± SEM.

Insulin was seen to increase PCOS preadipocyte proliferation. This was more predominant in PCOS cultures with significant increases seen by day 3 in both 10 and 100ng/ml insulin treatments (P=0.009 10ng/ml and P=0.008 100ng/ml). In 100ng/ml insulin treatment when examined significant increase was also seen in proliferation on day 5 against un-treated (*P<0.05).

Insulin and LH has been seen to enhance steroidogenic function in gonadal tissue through increased steroid biosynthesis [145, 299]. This may be as a result of increased proliferation under these conditions. As our later experiments would be examining steroid biosynthesis within adipocytes it was therefore necessary to explore this combined effect of insulin and LH across both groups. This may allow possible association in any later metabolic response results. **Figure 97** showed that the increased proliferation shown in non-PCOS adipocytes after treatment with 10ng/ml insulin was inhibited by the addition of 100ng/ml LH. These results further support the inhibitory response seen in our results from non-PCOS adipocyte LH treatments alone (**Figure 93**). However we continued to see no significant inhibition of PCOS adipocytes again supporting the lack of effect LH has on inhibiting PCOS adipocyte proliferation. However, the proliferative effects of insulin are slightly decreased with

the addition of LH as seen when comparing insulin against insulin + LH (see **Figure 95**, **Figure 96**, **Figure 97**). This may suggest that LH inhibition may be reduced rather than not exist in PCOS adipocytes.

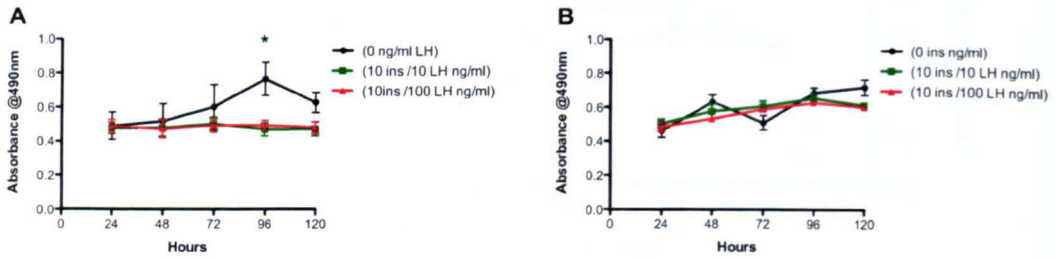

Figure 97. (A) **Non-PCOS preadipocyte proliferation under varied LH and Insulin treatments.** Non-PCOS preadipocyte cultures (n=5) were grown with and without a combination of LH and insulin treatments (10ng/ml insulin and either 10 or 100ng/ml LH) Proliferation was measured over 5 days using MTS assay. Significant **decreases** were seen on day 4 with both (10ins/10LH) and (10ins/100LH) (*P<0.05) when compared to un-treated non-PCOS cells. (B) **PCOS preadipocyte proliferation under varied LH and Insulin treatments.** Non-PCOS preadipocyte cultures (n=5) were grown with and without a combination of LH and insulin treatments (10ng/ml ins and either 10 or 100ng/ml LH) Proliferation was measured over 5 days using MTS assays. No significant variation was seen in proliferation between treated and un-treated cultures. Statistical analysis was done using unpaired *t-tests*. Results are mean ± SEM.

6.3.4 The influence of insulin and LH on CYP17 expression in non-PCOS and PCOS adipocytes

Insulin and LH have been shown to influence both proliferation and steroidogenic function in gonadal tissues, specifically ovarian granulosa and theca cells [145, 299]. In PCOS this has been hypothesized to be associated to hyperandrogenemia [145].

It has already been established that androgen synthesis comes from circulatory precursors within adipocytes with increased secretion from subcutaneous adipocytes when compared with omental adipocytes [191]. Our results have supported published findings that steroidogenic enzymes exist within adipocytes with the capacity to produce localized androgen synthesis. We have also examined levels of expression of the key enzyme CYP17 in both non-PCOS and PCOS SC adipocytes. Further to this we examined whether the hormonal treatments with PCOS would affect these levels and whether variation between non-PCOS and PCOS would occur. This would therefore add possible mechanisms of PCOS associated hyperandrogenemia.

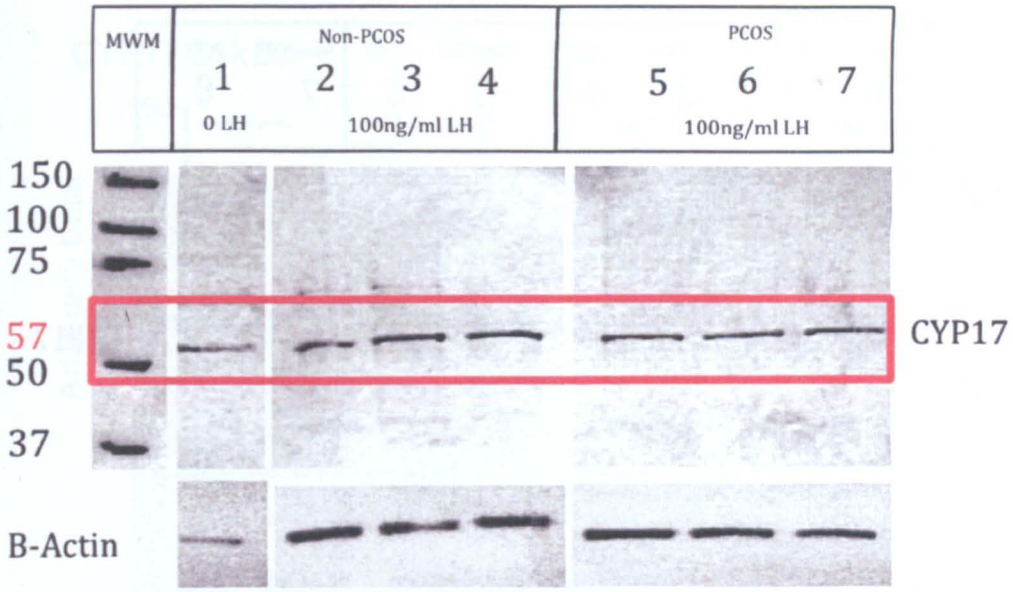

Figure 98. Western blot of CYP17 expression in non-PCOS and PCOS mature adipocyte (lane 1-8) after LH treatment. Mature adipocyte (n=3) were treated with 100 ng/ml LH *in-vitro* (Non-PCOS lane 1-3) (PCOS lane 4-6). Lanes were probed using CYP17a1 antibody. Exposure at 1240sec shows immunoreactive bands at 57kDa with no variation in band intensity. The blot was then stripped and re probed using β-Actin to control for protein loading. All blots were probed against CHO controls (not shown).

Firstly we examined the effects of LH on CYP17 expression in both non-PCOS and PCOS mature adipocytes (*see* **Figure 98**). Following normalization using β–Actin, no significant variation was seen between sample groups. With no effect seen in LH treatments we moved on to our second hormone of interest, insulin. Insulin treatments were examined individually. **Figure 99** showed paired samples; non-PCOS, PCOS adipocytes lysates following 0-1-10-100ng/ml insulin treatments (lane 2, 4). In non-PCOS samples CYP17 was seen to increase at around 10ng/ml treatments with no variation in expression with further increases to 100ng/ml. Similar expression was seen across non-PCOS and PCOS samples in the treatment range 10-100 with no significant variation in expression found. However PCOS samples showed increased CYP17 expression at lower insulin treatments when compared to non-PCOS (1ng/ml) and showed significant variation (***$P<0.001$) when quantification of WB band intensities against the β-actin was examined (see **Figure 100**).

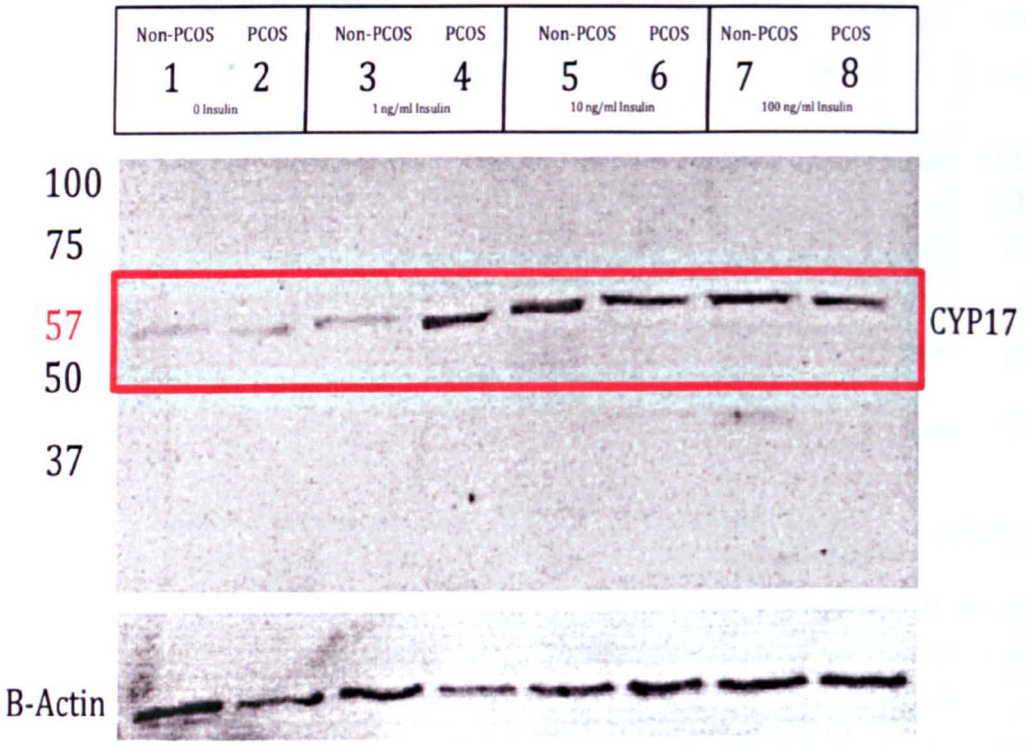

Figure 99. Western blot CYP17 expression in non-PCOS against PCOS mature adipocyte (lane 1-8) after varied insulin. Mature adipocyte were treated with varied doses of insulin *in-vitro* (insulin 0 (lane 1-2) 1ng/ml (lane 3-4) 10ng/ml (lane 5-6) 100ng/ml (lane 7-8). Lanes were probed using CYP17a1 antibody. Exposure at 640sec shows bands intensity at 59kDa with increases in insulin. Little variation was seen between non-PCOS (lanes 1, 3, 5, 7) and PCOS samples (lanes 2, 4, 6, 8). However increased CYP17 expression can be seen in PCOS (lane 4) at 1 ng/ml insulin treatment. The blot was then stripped and re probed using β-Actin to control for protein loading. All blots were probed against leucocyte negative controls (not shown).

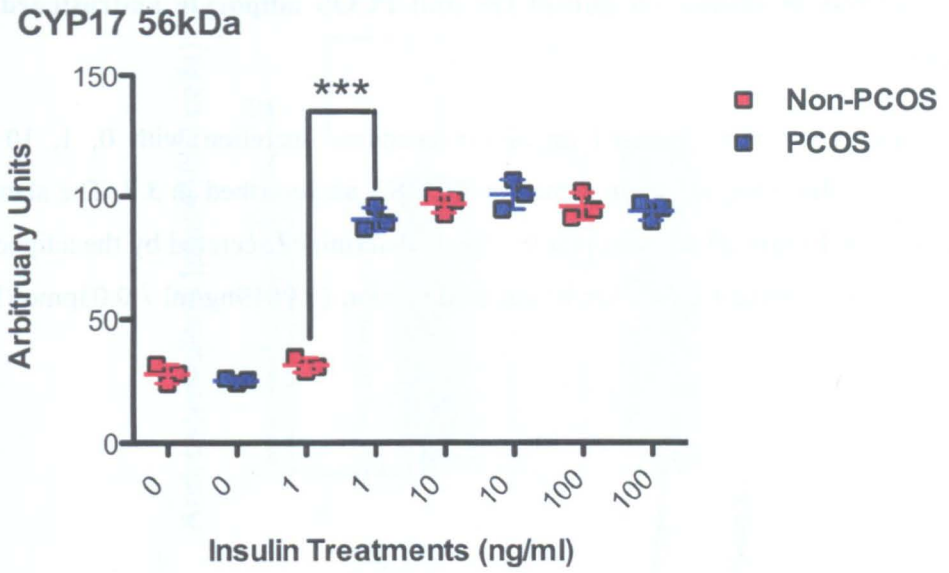

Figure 100. Summarising the densitometry representative of average expression of CYP17 in non-PCOS and PCOS mature adipocytes (n=3) under varying insulin treatments. Using β-Actin normalisation to compare levels of band intensity in western blot CYP17 expression (**Figure 99**) Densitometry examination of western blot results (**Figure 99**), showed significant difference were seen in expression of CYP17 with increases in insulin treatment of 1ng/ml (***P<0.001). Statistical analysis was done using one way ANOVA data expressed as means / ± SEM. No variation in CYP17 expression was seen between any other non-PCOS and PCOS insulin treatment groups.

6.3.5 Hormonal influences on non-PCOS and PCOS steroid secretion.

With suggested differences seen within proliferation and CYP17 expression between non-PCOS and PCOS adipocytes and with PCOS showing increased sensitivity to insulin treatments, we decided to examine whether the treatment levels used within our previous experiments would influence our determination of androstenedione secretion (*see Chapter 5*). Androstenedione secretion under controlled conditions, and following varied hormonal treatments of insulin and LH treatments were examined to determine levels across non-PCOS and PCOS mature adipocytes and whether any variations may exist.

6.4.5.1 Effects of insulin on non-PCOS and PCOS adipocyte androstenedione secretion

Our initial experiments focused on androstenedione secretion with 0, 1, 10 and 100ng/ml insulin using the androstenedione ELISA as described in *3.7*. The standard curve, seen in **Figure 35** showed that the levels determined secreted by the adipocytes fell within the standard curve for levels of detection (0.0019ng/ml / 0.03pmol/1000 cells).

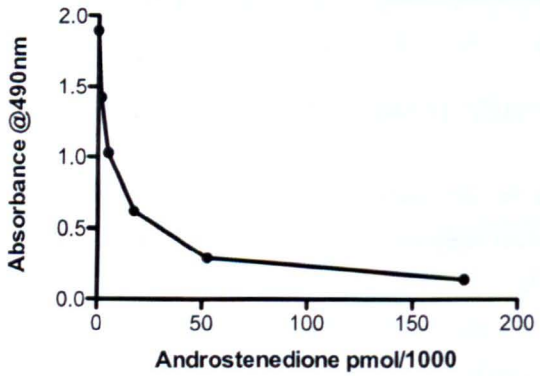

Figure 101. Standard curve showing ELISA androstenedione levels through measured absorbance. Known levels of androstenedione were measured using ELISA technique and levels of absorbance @490 taken to determine a standard curve.

We then compared androstenedione secretion from our two primary culture group's non-PCOS and PCOS adipocytes using the standard curve produced. **Figure 102** shows non-PCOS adipocytes (n=12) secreting significant androstenedione levels when compared to the non-secreting osteoclast negative control. The effect seemed to be dose dependant with increased androstenedione secretion at 10 and 100ng/ml when compared to no treatment (10 ng/ml *P<0.05 / 100 ng/ml **P<0.01).

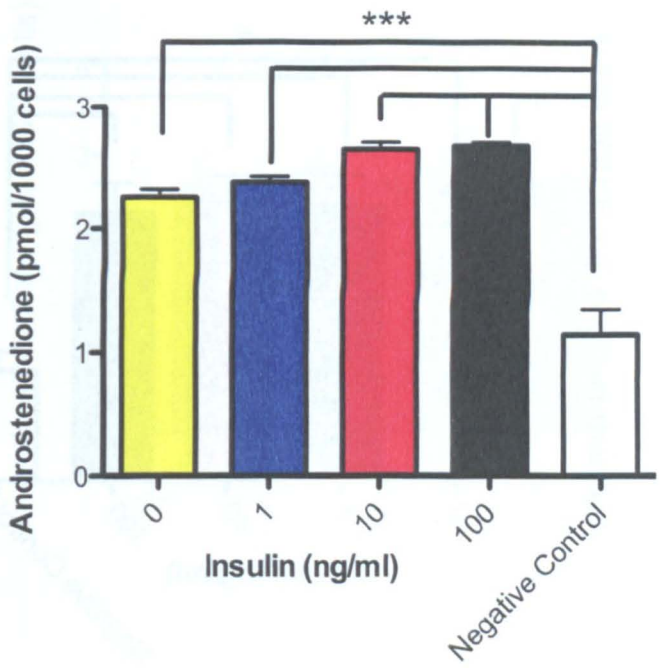

Figure 102. Androstenedione secretion (pmol/1000 cells) by non-PCOS adipocytes after varied insulin treatment. Non-PCOS mature adipocyte cultures (n=12) were grown after varied insulin treatments (1, 10, and 100ng/ml) for 3 days. Androstenedione levels were measured in conditioned media using ELISA. All measurements were compared to negative osteoclast controls using one-way ANOVA and *bonfferoni* post hoc test analysis and androstenedione levels found to be significant (***P<0.001) in all untreated and treated adipocyte cultures. Results are mean ± SEM.

Examination of PCOS adipocyte androstenedione secretion (n=9) after the same insulin treatments again showed significance against the negative control but had no variation across insulin treatments range. No variation was seen across any insulin treatment **Figure 103**.

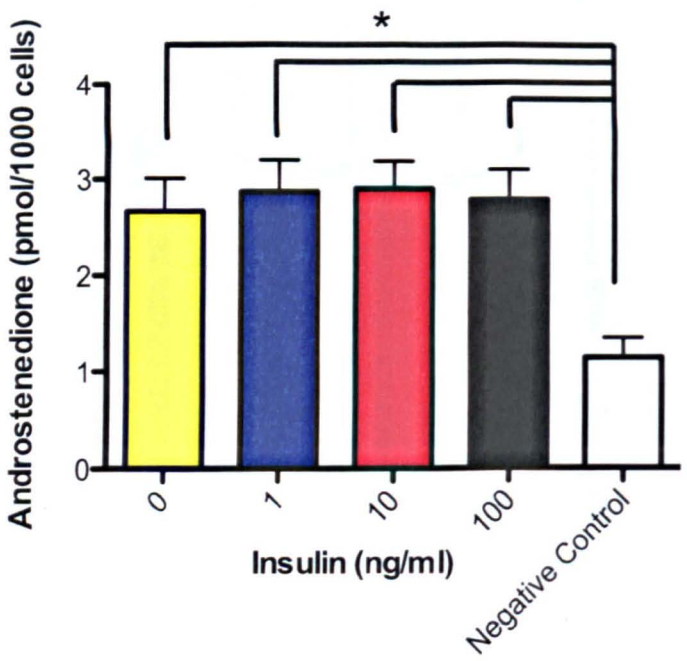

Figure 103. Androstenedione secretion (pmol/1000 cells) by PCOS adipocytes after varied insulin treatment. PCOS mature adipocyte cultures (n=9) were grown after varied insulin treatments (1, 10, and 100ng/ml) for 3 days and media examined for androstenedione levels using ELISA technique. All measurements were compared to negative osteoclast control cultures and androstenedione levels found to be significantly higher (*P<0.05)when examined using one-way ANOVA and *bonfferoni* post hoc tests. No significance was seen against the untreated cultures through any treatments.

Non-PCOS androstenedione secretion was compared against PCOS secretion under varied insulin treatments as seen in **Figure 104**. Statistical analysis showed no significant variation between groups however a trend does seem to exist between non-PCOS and PCOS androstenedione secretion that may suggest PCOS secretion is increased.

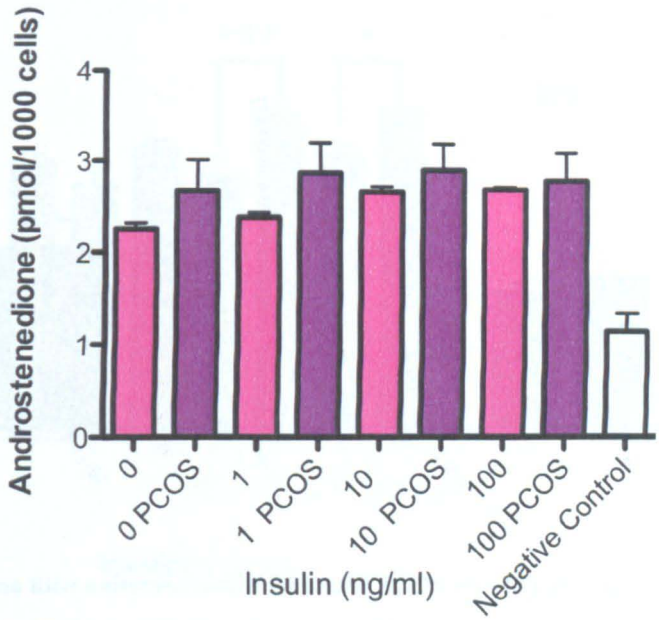

Figure 104. Comparison of non-PCOS and PCOS adipocyte androstenedione secretion after varied insulin treatment. Comparison of non-PCOS (n=12) and PCOS adipocyte (n=9) androstenedione secretion. No significance was seen within groups of insulin treatment using oneway ANOVA. Results are mean ± SEM.

With the possible influence insulin may therefore play in androgen synthesis we examine a possible signalling pathway through inhibition of PI3-K which has been shown to be involved in ovarian steroidogenesis [45]. **Figure 105** shows that no variation in either non-PCOS or PCOS groups was seen whether through insulin stimulation of androgen secretion or through inhibition of PI3-K pathway. Results however again show that an increase in androgen secretion was seen throughout all treatments in the PCOS group when compared to the non-PCOS.

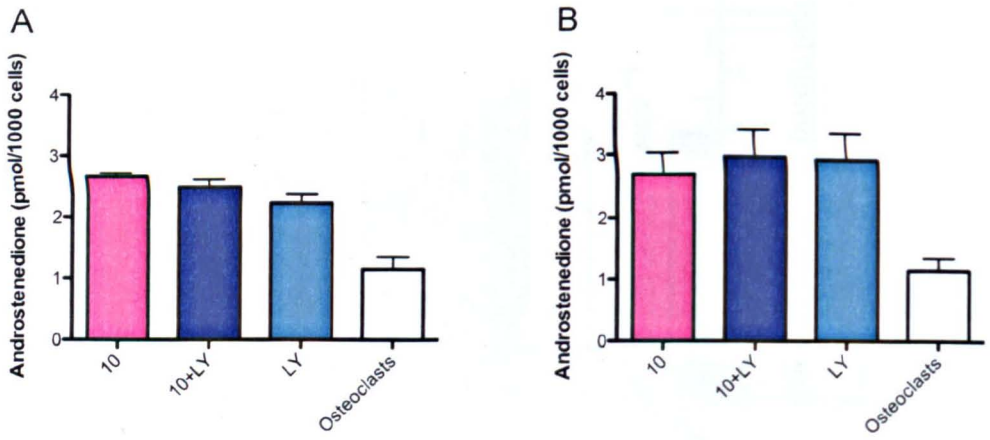

Figure 105. (A) Comparison of non-PCOS androstenedione secretion with and without inhibition of the insulin-signalling pathway. Non-PCOS adipocytes (n=12) were treated with insulin with and without the PI3-K inhibitor LY292004 and androstenedione secretion measured. No significant variation was found between treated and untreated cultures. **(B) Comparison of PCOS androstenedione secretion with and without inhibition of the insulin-signalling pathway.** Androstenedione secretion by PCOS mature adipocyte cultures (n=10) were also examined and no significant variation between levels of secretion were seen. Results are mean ± SEM

6.4.5.2 Effects of LH on non-PCOS and PCOS adipocyte androstenedione secretion

With augmentation of LH and insulin shown to increase androgen secretion in ovarian theca cells [91] we treated the non-PCOS cultures with a combination of treatments and measured the effects on androstenedione levels found in media following 3 days maintenance. A trend was seen throughout all treatments with the addition of LH to insulin treatments showing reduced androstenedione levels suggestive of inhibition. Furthermore significant increases were seen on days 4 (***$P<0.001$) and day 5 (*$P<0.05$).

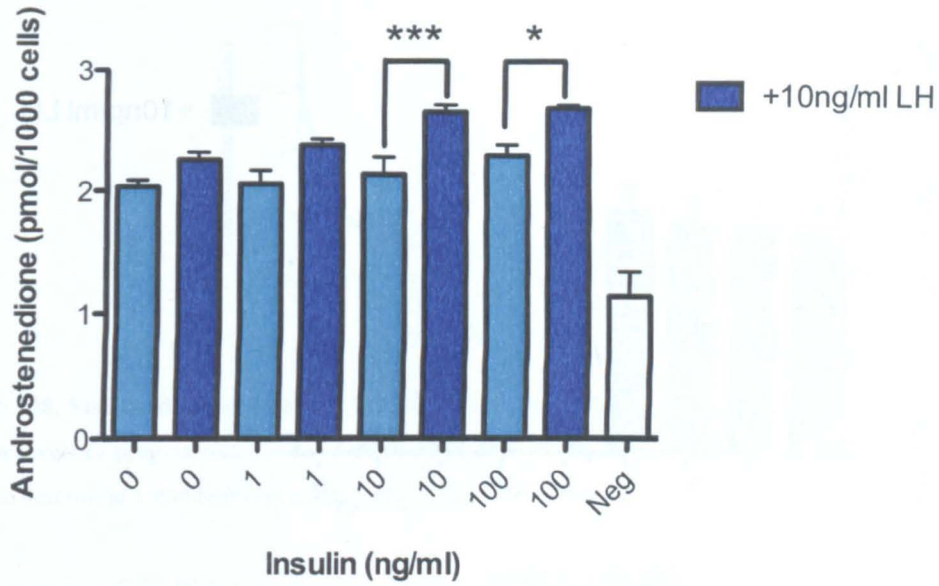

Figure 106. Comparison of non-PCOS mature adipocyte androstenedione secretion with and without varied insulin and LH treatments. Androstenedione secretion was measured from non-PCOS mature adipocyte (n=12) after varied insulin secretion (0, 1, 10 and 100ng/ml) and with or without 10ng/ml LH treatments. Results are mean ± SEM .Significant increases were seen between androstenedione levels from 10ng/ml insulin treatments against 10ng/ml with 10ng/ml LH treatments (***P<0.001). Significant increases in androstenedione secretion were also seen within 100ng/ml insulin treatment against 100ng/ml with 10ng/ml LH treatments (*P<0.05) when examined using one-way ANOVA and *bonfferoni* post hoc tests.

In PCOS cultures we saw minimal effects at low insulin and LH treatments and minimal reductions in androstenedione secretion at the higher insulin and LH treatments in comparison to non-PCOS. It should be noted that although no significant differences were seen within any treatments in the PCOS groups the trend was the same as seen in the non-PCOS groups (**Figure 103**). This may suggest reduced sensitivity if examined further.

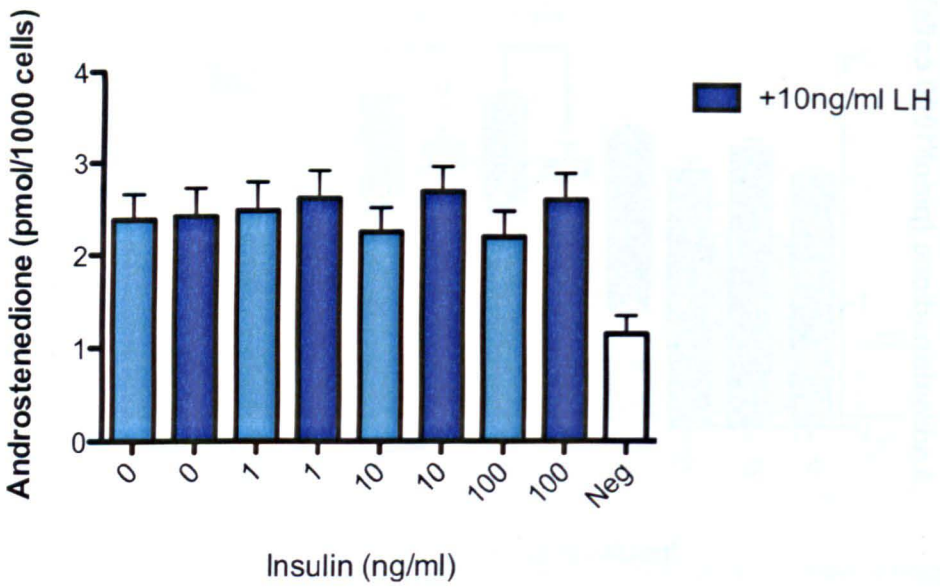

Figure 107. Comparisons of PCOS mature adipocyte androstenedione secretion with and without varied insulin and LH treatments. Androstenedione secretion was measured from PCOS mature adipocyte (n=9) after varied insulin secretion (0, 1, 10 and 100ng/ml) and with or without 10ng/ml LH treatments. Results are mean ± SEM. No significant variations were seen between treated or untreated groups.

6.4.5.3 Effects of Insulin and LH on non-PCOS and PCOS adipocyte progesterone secretion.

Using a progesterone ELISA (*see 3.7*) we examined progesterone secretion in both non-PCOS and PCOS adipocytes. Known levels of progesterone were examined using the same ELISA and absorbance measured to create a standard curve. All results were obtained using this standard curve showing values falling within the ELISA range of capability.

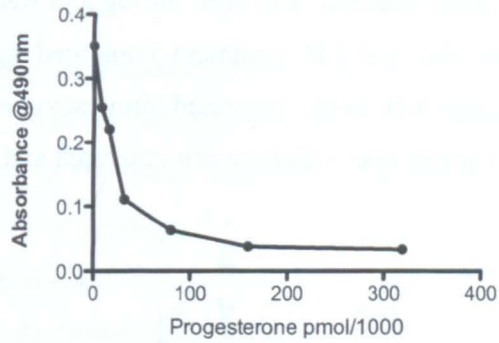

Figure 108. Standard curve showing ELISA progesterone levels through measured absorbance. Known levels of progesterone were measured using ELISA technique and levels of absorbance @490 taken to determine a standard curve for which all results could be measured by.

Examination of ELISA results taken from non-PCOS adipocytes treated with varying doses of insulin with and without additional LH treatment (100ng/ml) **Figure 109** showed progesterone secretion occurred against negative osteoclast controls.

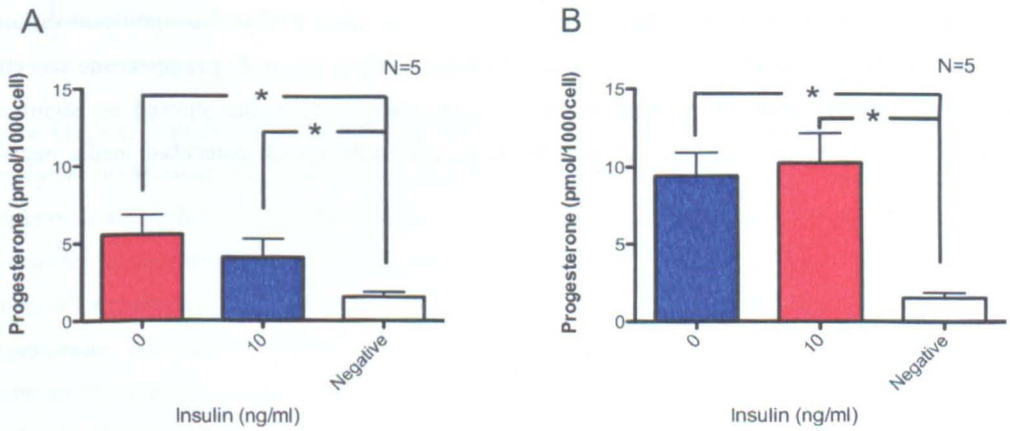

Figure 109. (A) Non-PCOS mature adipocyte progesterone secretion with varied Insulin treatment. Insulin treated and un-treated non-PCOS mature adipocytes (n=5) media was examined for progesterone using ELISA technique. Results are mean ± SEM. All levels were significant against the control (*P<0.05). No significant variation between treated and un-treated was seen. **(B) PCOS mature adipocyte (n=5) progesterone secretion with varied Insulin treatment.** PCOS mature adipocyte media showed no significant variation in progesterone secretion between treated and un-treated. All samples were examined against osteoclast media negative control and one-way ANOVA and *bonfferoni* post hoc tests.

No significant variations occurred across treatments in both non-PCOS and PCOS groups. However examination of PCOS progesterone secretion showed increased

levels. Although no significant increase was seen throughout treatments an increased trend was seen within insulin and LH treatment combined against insulin alone. Although the results suggest LH alone increased progesterone secretion in PCOS samples, no significant variation was seen between untreated and LH treated secretion.

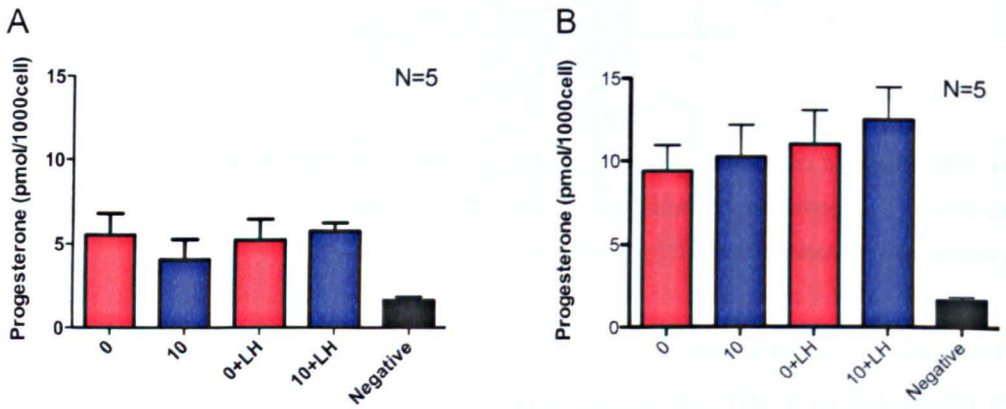

Figure 110 (A) Non-PCOS mature adipocyte progesterone secretion with varied Insulin and LH treatment. Insulin and LH treated and un-treated non-PCOS mature adipocytes (n=5) media was examined for progesterone using ELISA technique. Results are mean ± SEM. No significant variation between treated and un-treated was seen. **(B) PCOS mature adipocyte (n=5) progesterone secretion with varied Insulin and LH treatment.** PCOS mature adipocyte media showed no significant variation in progesterone secretion. All samples were examined against osteoclast media negative control.

6.4.6 Effects of progesterone on non-PCOS and PCOS adipocyte proliferation.

With results suggesting the presence of progesterone secreted by adipocytes into the media we decided to examine whether localized secretion would have an effect on adipocyte proliferation at the levels measured. The progesterone treatments chosen were therefore kept within the range 0, 0.25 and 0.1 ng/ml (5-15 pmol/1000 cells) and MTS assay used to examine 5 day proliferation. Results showed that within 24hr of incubation significant decreases in proliferation were seen in comparison to untreated groups in both non-PCOS and PCOS cultures **Figure 111**. Cell morphology showed rounding and large numbers of cellular debris suggestive of cell death.

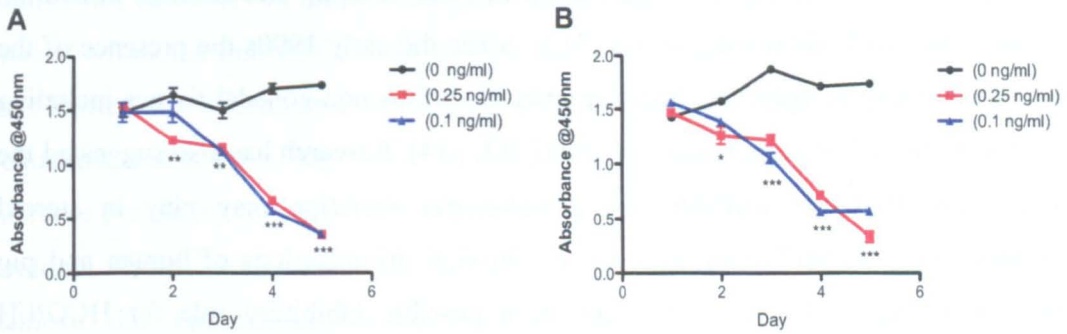

Figure 111. (A) Non-PCOS 5 day proliferation after varied progesterone treatments. Non-PCOS adipocytes (n=3) were examined **after** varied progesterone treatments (0.25 and 0.1ng/ml) for proliferation over 5 days using MTS assay. Results are mean ± SEM. Significant variation was seen by day 2 under 0.25 ng/ml (day 2/3 **P<0.01 day 4/5 ***P<0.001). Significant variation was seen by day 3 under 0.1 ng/ml (day 3 **P<0.01 day 4/5 ***P<0.001). **(B) PCOS 5 day proliferation after varied progesterone treatments.** PCOS adipocytes (n=3) were examined **after** varied progesterone treatments (0.25 and 01 ng/ml) for proliferation over 5 days using MTS assay. Significant variation was seen by day 2 under 0.25 ng/ml (day 2 *P<0.05 day 3/4/5 ***P<0.001). Significant variation was seen by day 2 under 0.1 ng/ml (day 2 *P<0.05 day 3/4/5 ***P<0.001) using one-way ANOVA statistical examiation.

6.5 Discussion

6.5.1 Expression of LH receptor in non-PCOS and PCOS adipocytes

The aim of this chapter was to investigate the expression the LH receptor within pre/mature adipocytes as first examined by Dos Santos *et al.*, (2007) and to also determine what effect PCOS may have on LHR function in adipocytes [300]. To achieve this we studied the possibility of variation between non-PCOS and PCOS LHR expression as well as variations that may occur across stages of adipocyte development.

The LH receptor is recognized as a trans-membrane receptor and is found in ovarian thecal cells [311] and uterine serosa [312]. Since the early 1990s the presence of the HCG/luteinising receptor has been reported in various non-gonadal tissues including neuronal, thyroid and adrenocortical cells [313, 314]. Research has also suggested the importance HCG/LH receptor and gonadotropin secretion may play in steroid hormone metabolism within these tissues through investigations of human and pig mammary glands. These studies indicate a possible inhibitory role for HCG/LH within mammary carcinogenesis and the growth of breast tumours [315, 316]. This research therefore points to the existence of an LH mechanism in non-gonadal tissue; and a subsequent relationship in steroid hormone metabolism which to date has been well documented in gonadal tissues [317-319]. Our findings support the localisation of the LHR within non-PCOS adipocytes with immunofluorescence and western blotting techniques showing positive results at both preadipocyte and differentiated adipocyte development. Our western blot results also showed a double banding effect, which may be a due to glycosalation/phosphorylation. Lysates studies into ovarian LHR have shown that prolonged receptor occupancy can result in conformational changes and phosphorylation which may also explain our findings[320]. Our findings are supported by Dos Santos *et al.*, (2007) who suggested there maybe variation in LHR expression between pre- and mature adipocytes which we examined further and confirmed [300]. Interestingly the reverse is seen in published studies into other hormonal agonists including insulin where IRS-1 and 2 is up regulated following differentiation of adipocytes [321].

These results may therefore suggest a central role of LH in post differential activity. The results obtained through immunofluorescence were useful in determining the presence and location within the cell membrane of the LH receptor stressing the possible involvement of external regulation. These results should however be seen as more indicative than conclusive. They allowed us to justify further extension into the presence and regulatory mechanisms of LHR expression and function through similar findings within our western blots results. Furthermore, due to the difficulty of working with the fragile mature adipocytes it was necessary to optimize the fixation aspect of the immunofluorescence protocol to reduce cell damage as seen in Chapter 4. This improved the clarity of the images. It was observed that due to the level of confluence necessary to allow maturation of the adipocytes there was an increase in 'noise' within the field of view, which was also prone to photo-bleaching and viewing was limited to within an hour of the washing procedure. From this we determined that reduction in dilution of the primary antibody and a reduction in cell number would increase the clarity of images and reduced the bleaching effect. Ideally we required a protocol that allowed maturation in smaller cell numbers. Although we failed to achieve this optimisation, further work would have included smaller growth environment with reduced cell numbers and using fibronectin to support cell adhesion [322]. We should also consider quantification of this technique with a constant exposure time this can be achieved using a suitable software package and a micro spectrofluorimeter as described by Casperson *et al* (1965) . The semi quantification of western blot was however a more suitable protocol and allowed the findings to be supported. Due to time constraints we were unable to examine this further utilizing RT-PCR as we had intended and the results shown were sufficient in supporting expression in order to justify the further work into hormonal activity.

Further to this work we were able to examine results within PCOS primary cultures, to determine whether any variation exists across development. In conclusion we found no differences between non-PCOS and PCOS LHR expression at any stage.

The determination of other hormone receptors in adipocytes and their subsequent ability to communicate resulting in an adipocyte response is becoming more apparent. Research by Hansen *et al.*, (1998) showed growth hormone (GH) exerting an influential adipogenic effect on preadipocytes through its own receptor and animal

studies have shown that an inhibitory effect on preadipocyte differentiation exists under increased GH conditions [323]. Our evidence to support the existence of the LHR receptor in both non-POCS and PCOS, adipocytes enhances these findings through its suggestion of alternate hormonal influences on adipocyte metabolism and regulation. Combined with further examination, these results may go someway to suggest an area associated with endocrine dysfunctions seen in conditions such as metabolic disease and PCOS where hyper secretion of steroid messengers leads to exaggerated adipocyte responses.

6.5.1.2 The effects of Insulin and LH on LH receptor in non-PCOS and PCOS adipocytes

With results suggesting the presence of LH receptor in PCOS adipocytes, many areas of research become open to examination, such as responses to physiological and hyper-physiological hormonal levels. With hormonal dysfunction being one of the key characteristics associated with PCOS, specifically hyperinsulinemia and hyper secretion of LH, we examined the receptor response from varied insulin and LH treatments in the context of the condition. All experiments were carried out on mature adipocytes and the treatment ranges chosen were those used in gonadal steroidogenic studies specifically Gilling-Smith *et al.*, (1994) and Munir *et al.*, (2004) [45, 91]. These treatments ranges had been previously shown to exhibit optimal insulin and LH responses. Experiments on non-PCOS and PCOS cultures showed reduced LHR expression following LH treatments. Our western blot results showed LHR expression in untreated cells but should ideally have been run on separate blots to avoid saturation during exposure. By removing the insulin treatment we could have increased the exposure time without saturation occurring and therefore further support the influence found. This may have allowed us to see the range of LH treatments used has a more sensitive inhibitory effect or whether 10-100ng/ml of LH has the same inhibitory effect shown from our results. Further to this a more quantitated analysis could be achieved through RT-PCR allowing the exact levels of variations through mRNA analysis. This would give us an idea of variation at another level of regulation they may refute or support our current analysis.

The inhibitory effect of LH shown on LHR is similar to that seen by Yamamura *et al.*, (2000) who examined granulosa cells in chickens. Yamamura showed that both LHR and FSHR mRNA was down regulated by LH, progesterone and estradiol during the ovulatory cycle and concluded that steroid mediation of both receptors was possible both directly and indirectly through their interaction with LH stimulation [324]. It is therefore feasible that adipocytes show similar traits in LH mediated expression of its own receptor limiting a downstream cellular response yet to be determined.

Results presented here in, also showed an increased response in LHR expression with increasing insulin treatments in both non-PCOS and PCOS samples. The first increase was seen with the addition of a 10ng/ml treatment and remained similar at 100ng/ml suggesting 10ng/ml was the optimum treatment required for the greatest response in LHR expression within this range. This did not vary across PCOS samples and we can conclude from these results that the increase in LHR expression due to increases in insulin treatments was the same for non-PCOS and PCOS adipocytes Research has established the positive effects of insulin and insulin like growth factor (IGF) on increasing LHR expression in gonadal tissues such as theca and granulosa ovarian cells [325]. Examination into non-gonadal tissue has also been examined, studies into leydig tumour cells by Zhang *et al.*, (1998) showed similar results with the addition of IGF-1 [326]. Under the treatment range 0.1–100ng/ml, they found that levels around the 20-30ng/ml were sufficient to cause an increase in LHR mRNA. Furthermore they found that an increase in IGF-1 levels up to 100ng/ml did not lead to further increases in LHR [326]. From our results we can determine a possible agonistic response in LHR expression exists within both pre and mature human adipocytes in response to insulin, suggesting an involvement in adipocyte metabolism and sensitive *in-vitro* to 1 ng/ml insulin with no variation up to 100 ng/ml.

6.5.2 The effects of LH and Insulin on non-PCOS and PCOS adipocyte proliferation.

Although the hormonal effects of both LH and insulin on proliferation in both gonadal and non-gonadal tissues have been examined, research into adipose is yet to be examined. Furthermore research into PCOS adipocytes proliferation under these

conditions is yet to be observed and with recent discovery of the adipocyte LH receptor [300] it is of particular interest with regard to the condition.

It is widely accepted that ovarian cell proliferation is increased during LH secretion in the follicular phase of the menstrual cycle. This allows greater androgen synthesis through stimulation of increased theca cell production as well as encouraging androgen secretion and differentiation [299, 327]. The importance of LH in regulation of cell proliferation has also been seen in other tissues including Leydig cells where, conversely, Srirman *et al.,* (2000) by monitoring the levels of proliferating cell nuclear antigen (PCNA) with the addition of LH, showed a significant decrease in proliferation [328]. From this we can see that LH plays a key role in regulating proliferation in certain tissues and also the importance this regulation plays in hormone metabolism within gonadal tissues.

Although adipocyte metabolic response to LH has yet to be examined thoroughly, our results suggest a proliferative response exists and that the addition of LH to non-PCOS preadipocytes leads to a significant decrease in proliferation. More specifically results from our assay indicated complete inhibition of proliferation over the five day assay.

Our comparative PCOS model however showed different results and lacked inhibition of proliferation within the same insulin treatment range. From this we can suggest that a possible lack of LH sensitivity exists within PCOS adipocytes, that affects the LH regulatory mechanism of a proliferative response. Similarly this effect is seen in PCOS gonadal cells under the influence of insulin and it is understood that an association with insulin resistance exists in PCOS that has been linked to hyperinsuilnemia and hyperandrogenemia. This may therefore also exist with LH.

Interestingly the responses in adipocytes seen with the addition of insulin were not as potent with regard to proliferation as would be expected. Studies have shown that insulin increases the proliferative response of adipocytes within the doses we tested [310, 321]. No significance was seen between our non-PCOS samples and insulin treatments which suggested less potent response than previously published [310]. It should, however, be noted that the limited sample numbers used in this study may account for this variation as shown through the level of error in findings. A reduction

in the error found may therefore show significance between the control and both insulin treatments which showed similar results and pointed to a possible increase over the 0 treatment. Although MTS assays focuses on mitochondrial activity, allowing a good measure of viable proliferation, the observation would be better supported using additional techniques such as evaluation of cell death through lactate dehydrogenase (LDH) release. This is routinely used as a measure of tissue breakdown through its involvement in pyruvate to lactate conversion [329]. Time restraints meant this was not an option.

It should also be noted that MTS assay is a measure of metabolic activity through its cellular conversion to formazen. Treatments used may have an effect on this activity and therefore affect proliferation results. Huang *et al.,* (2004) reported that the albumin content in serum may reduce absorbance by as much has 50% [330]. Although these conclusions are still under examination and vary on repeats, results taken from MTS/MTT assays can be supported through cell counting.

This study did allow us to see differences between non-PCOS and PCOS adipocytes. Firstly, growth patterns varied between the two groups with PCOS reaching the same level of proliferation as non-PCOS. However, non-PCOS cell numbers where seen to decrease over the longer term when compared to PCOS. This may be attributed to PCOS cells clumping or stacking which fails to occur in non-PCOS cells. By increasing the MTS assay period from 5 days we would be able to establish whether PCOS can exist in this state for longer as when this begins to occur in non-PCOS cells, numbers begin to reduce as seen by day 5 in our results. Our results are not conclusive enough to report PCOS cells can exist in a different growth state than non-PCOS although evidence may suggest this. Secondly, the PCOS adipocyte response to insulin showed a greater sensitivity with increased proliferation seen. This may be a PCOS adipocyte dysfunction that should be examined further. Our findings also showed further increases in proliferation occurred on day 5 within our largest insulin treatment group suggesting that hyperinsulineamic conditions may play a greater role on the effect on adipocyte metabolism.

Finally the results seen using insulin treatment further supported the capacity of LH to inhibit proliferation and all effects seen in non-PCOS with the addition of insulin were reduced with the addition of LH. Although such a strong inhibitory effect was

once again not seen in the PCOS samples we did find that the large increase in proliferation found with the addition of insulin treatments alone was reduced with the addition of LH. This may also suggest that the defect that may exist in PCOS preadipocytes is linked to sensitivity rather than a complete abolition of the effect of LH. With future work I may examine this further and an increased treatment range would be considered both increasing and decreasing the dosages of LH used to determine actual sensitivity.

6.5.4 The influence of insulin and LH on CYP17 expression in non-PCOS and PCOS adipocytes

With the possibility that proliferation in adipocytes is influenced by hormonal stimulus we began to look into areas of associated metabolic response particularly those associated to PCOS.

Studies have shown IGF-1 has positive effects on steroidogenic enzyme expression and increased IGF-1 influence on both adrenal cortical cells and leydig cells have been shown to up-regulate 17 alpha-hydroxylase/C17-20 lyase, cholesterol side-chain cleavage, and type I 5 alpha-reductase (5 alpha R-1) all known to play vital roles in androgen synthesis. This has also been seen in ovarian cells, with studies by Munir *et al.*, (2004) showing an increase in steroidogenic enzyme expression in theca cells. Our examination into the steroidogenic enzyme CYP17 further supports these findings and our results (as seen in Chapter 5) showing the presence of CYP17 in adipocytes allowed us to examine its expression under the influence of insulin and LH treatments [45]. Initial experiments comparing CYP17 expression across non-PCOS and PCOS samples showed no significant variation, however when compared following insulin treatment PCOS cells showed signs of increased sensitivity to insulin at lower treatments than those seen in non-treated, suggesting a defect may exist. With the prevalence of insulin resistance within PCOS this finding seemed paradoxical. Similar hypotheses exist within ovarian theca studies with suggested molecular defects of CYP17 expression under increased insulin levels leading to increased androstenedione synthesis within PCOS [45]. These results should only be considered as a pilot study. Lysate examination was limited (n=3 *vs.* 3) and we attempted to keep results consistent to high BMI, hyperinsulinemia groups leaving

room for error. In order to further validate /support these findings it would be necessary to increase the sample size. In further work I would also use direct homogenization of biopsies to eliminate any cellular metabolic variations associated to *in-vitro* treatment. The availability of samples meant that culturing was however required for all experiments to be undertaken. These results may however require further repetition with increased samples and would be more valid utilising examination of CYP17 mRNA under RT-PCR experiments.

6.5.5 Effects of Insulin and LH on non-PCOS and PCOS adipocyte hormone secretion.

Following examination and determination of androstenedione present within our media harvest and shown within both our non-PCOS and PCOS primary adipocyte culture groups (*see 5.4.7*) we ran the same protocol under varied hormonal treatments to determine if any influence on levels of secretion existed. The same ELISA was used alongside intra / positive theca culture control and negative osteoclast culture media harvests (*see 3.6.1*). Both groups were found to have increased levels of androstenedione significant against the negative osteoclast under all treatments. The addition of our insulin treatment range within non-PCOS cultures showed an increase in androstenedione secretion at 10–100ng/ml with no variation between these treatments. From this we can therefore suggest insulin influences androstenedione metabolism in non-PCOS mature adipocytes at this treatment level causing an increase in secretion over 3 days. We took these results further and examined a possible pathway in which insulin could be affecting androstenedione synthesis similar to that seen in ovarian androgen synthesis. The protocol used was similar to that used by Munir *et al.*, (2004) and used a PI3-K inhibitor (LY294002) in combination with insulin treatment levels previously shown to increase androstenedione levels. This allowed us to examine the effects insulin had on levels of secretion (*see 3.7*). The results in both non-PCOS and PCOS samples showed no variation occurred between inhibited and un-treated samples. This would suggest that if insulin was stimulating androstenedione secretion it is not working through the PI3-K pathway. Results taken for the PCOS samples did however show slight discrepancy in samples treated with the inhibitor alone. We found androstenedione levels in these samples were similar to all insulin treated and untreated samples. Although this result

supports lack of influence on androstenedione levels found in all the PCOS samples, we did expect this to be reduced in the inhibited group due to the reported toxic effect of LY294002 [331]. We may be able to associate this to the sample size and the resultant room for error this incurred. Future work would include increase in these numbers to support these findings. Although future work may benefit the study through use of radio immune assay (RIA) examination, the main benefit would be the level of sensitivity. The ELISA used was chosen for its ability to recognise very low levels of androstenedione and therefore RIA would be more supportive than used as an alternative.

Our further examination into the progesterone precursor required for localised androstenedione synthesis, also showed supportive evidence of a hormonal influence and the pathway involved in androgen synthesis. Although no significant difference in progesterone level were found under hormonal treatments the results showed a possible trend toward decreased progesterone synthesis at 10 ng/ml insulin. This is the same level required to cause androstenedione levels to increase. If a pathway utilising progesterone existed in the conversion to androgen then a possible reduction may be seen at this point. The lack of significance may be accounted for again through lack of sample size. We should also consider that due to restrictions, we were limited in examination of our insulin range for progesterone and only examined 10 ng/ml as this was the lowest level shown to cause androstenedione increase. With more time we could have examined a greater range to see if this effect increased. Examination of the PCOS sample may also support this hypothesis as the lack of effect of insulin on androstenedione secretion seen in our PCOS samples may lead to a stable or increased level of progesterone in this situation, which was found on examination. This may also suggest either increased progesterone though lack of synthesis or presence of a defect in PCOS adipocytes leading to increased progesterone secretion or synthesis. If we compare this to the results we found with our androgen secretion then an increase trend or at least a similar level of androgen secretion would point to no less use of progesterone within the hypothesised pathway. We could therefore speculate that if an increased level of androgen is being synthesised then the increased level of progesterone is linked. Further examination is required and as mentioned examination of progesterone levels within increased sample numbers through RIA would test the hypothesis.

Although brief examination of the results comparing non-PCOS and PCOS progesterone suggested an increase in progesterone existed in PCOS adipocytes, no significant variation was found. The error bars shown and sample size used suggest further examination is required again with a larger sample groups. This is also seen when comparing the influence of LH and insulin combined where a definite trend is seen not only increasing with the addition of insulin in both non-PCOS and PCOS more predominantly in PCOS, but also increasing even further with the addition of LH. This does show significant increases within our PCOS samples with insulin and LH combined giving greater secretion of progesterone over insulin alone. These results point to a definite augmented effect of LH and insulin combined in PCOS samples and with further examination this may be the case within our non-PCOS samples although to a lesser degree. If this were the case it would suggest that the combination of insulin and LH not only had a greater effect on progesterone synthesis but that PCOS adipocyte were more sensitive to the action. If we were to determine that progesterone was active in adipocyte androgen synthesis pathway and that PCOS cells are also producing increased levels of androgen then the increased levels associated to a combination of LH and insulin would support the increased androgen secretion. This could easily be associated with conditions found in PCOS with symptomatic hyperinsulineamia and hyper secretion of LH acting to increase androgen synthesis within adipocytes due to a possible defect within PCOS adipocytes. Furthermore our results in examining androstenedione secretion showed significant increases in non-PCOS samples through augmented LH and insulin treatments and although no significance was seen within our PCOS samples a trend was noted.

6.4.6 Effects of progesterone on non-PCOS and PCOS adipocyte proliferation.

Various studies have shown that progesterone may increase adipocyte accretion [288, 332, 333] and it has been theorized this is through increased lipoprotein lipase (LPL) the enzyme responsible for lipolysis [334]. Although controversy exists, recent studies by Zhang *et al.*, 2008 have argued this not be the case, showing too many inconsistencies within results [335]. Further research into the influence of progesterone within proliferation of 3T3-L1 preadipocytes by Monjo *et al.*, 2004 has shown a significant positive effect at similar treatment levels to those in our study

[239]. However this was examined within omental adipocyte samples and they also report that the same examination into brown adipocytes showed the opposite effect occurs possibly through down regulation within the androgen receptor to which the study found association [239]. Furthermore progesterone has been shown to reduce the estradiol-induced proliferation of breast epithelial cells as well as endometrial proliferation *in-vivo,* and inhibition of proliferation of vascular smooth muscle has been reported [336]. Examination of our non-PCOS and PCOS primary cultures showed that a definite decrease in proliferation occurred at progesterone levels similar to those seen within our media harvests. Although limited by sample size our pilot study allows interesting information in this broad area of examination and may suggest that localized progesterone secretion may also be inked to a mechanism of regulation associated with proliferation.

7.0 HORMONAL INFLUENCES ON THECA CELL FUNCTION IN NON-PCOS AND PCOS WOMEN

7.1 Introduction.

Polycystic ovarian syndrome is a disorder of diverse clinical and biochemical symptoms ranging from anovulation to hyperinsulinemia [337-339]. Typically PCOS is associated with high circulatory LH and testosterone levels [144, 302]. Although evidence exists as to elevated adrenal androgen secretion contributing to hyperandrogenemia in PCOS patients [340, 341], whether this is through thecal hypertrophy or dysfunction leading to hyper secretion remains to be fully supported.

Steroidogenic activity within the ovaries is dependent on the collaboration of two cell types; granulosa and theca. Androgen biosynthesis occurs predominantly within the ovarian theca cells and contributes to around 50% of overall physiological androstenedione secretion [342]. The excess androgen synthesis found within PCOS is thought to relate to excess ovarian biosynthesis; although the exact underlying aetiology of the syndrome remains largely unexplained a significant body of evidence suggests that an excess of ovarian androgen production remains central in the pathogenesis of PCOS [119, 339]. Three possible mechanisms for this androgen hyper-secretion have been postulated including an intrinsic functional thecal defect, namely thecal hypertrophy; hyperinsulinemia resulting from insulin resistance, which is common in women with PCOS, or pituitary LH hyper secretion resulting in excessive thecal stimulation. Although, several publications exist on thecal function, in normal and polycystic ovaries including *in vitro* and *in vivo* studies [343-345], identification of a primary defect in PCOS is yet to be determined. In particular, the exact cellular mechanism of action of insulin in the theca cell remains to be fully understood.

Despite the prevalence of insulin resistance in women with PCOS [185], several studies have shown insulin receptors to be normal in structure [346], quantity and insulin binding affinity in these women [172]. It is therefore, now recognized that

insulin resistance in PCOS is due to post-receptor defects in the insulin-signalling pathway.

It has been clearly shown that insulin acts on theca cells through its own receptor [45, 157]. Despite insulin resistance in women with PCOS, ovarian theca cells show hypersensitivity to insulin resulting in excess androgen production. This paradox could be explained by selective defects of insulin sensitivity, which affects metabolic, but not mitogenic insulin signalling pathways [347, 348]. Another explanation for this paradox is that defects in insulin sensitivity are tissue-specific with resistance in peripheral (skeletal and adipose) tissues and hypersensitivity in ovarian theca cells [99]. In support of this hypothesis, a recent study has shown cell-specific alterations in insulin receptor substrate (IRS) protein concentrations in theca cells from polycystic ovaries that are consistent with an exaggerated amplification of the insulin signal [70].

Data on the mechanisms regulating androgen production distal to IRS are limited and conflicting. Research involving theca cells from normal ovaries has suggested phosphatidylinositol-3-kinase (PI3-kinase) (but not mitogen-activated protein kinase [MAPK]) as a possible insulin signalling mediator for the stimulatory effects of insulin on 17α-hydroxylase (CYP17), the enzyme involved in androgen biosynthesis, [45]. Alternately Poretsky *et al.,* 2001 found that insulin-mediated increase in steroidogenesis in granulosa lutein cells of normal ovaries is independent of PI3-K pathway [68]. No studies have been conducted on PI3-K in polycystic ovaries. The role of PI3-K pathway therefore remains uncertain in normal and polycystic ovaries.

Another area of uncertainty is whether insulin stimulates ovarian steroidogenesis independently or through augmentation of LH stimulated androgen synthesis by interacting with LH-induced cAMP. Nestler *et al.,* (1998) demonstrated that insulin alone stimulated and increased testosterone production by cultured theca cells from normal (4 fold increase) and polycystic ovaries (13 fold increase) [349]. In contrast to this Munir *et al.,* (2004) found that insulin alone was not able to stimulate 17α - hydroxylase activities in cultured theca cells from normal ovaries, but required activation of cAMP by adding forskolin [45]. Given this we sought to examine whether this same finding occurred within PCOS theca cells or whether a dysfunction

existed that might lead to an understanding of increased androgen synthesis seen within PCOS ovarian theca cells.

Clinical studies by Barnes *et al.*, (1989) and Erickson *et al.*, (1985) suggested that the key to dysregulation is related to the steroidogenic enzyme CYP17, and with results from Munir *et al.*, (2004) showing that expression of CYP17 is increased following augmentation of insulin and LH in non-PCOS thecal cultures [42, 45, 134, 340], further examination using cultures prepared from the theca of PCOS women is warranted.

In addition to these studies Gilling-Smith *et al.*, (1994) examined androstenedione secretion under varied LH treatments within non-PCOS and PCOS theca cells and found significant increases did exist between basal and LH-treated PCOS cells [91]. A combined effect is yet to be examined in PCOS cells with regard to effects on androstenedione secretion but all these results point to variations existing within the PCOS thecal cell steroidogenic pathway. More specifically research by Munir *et al.*, (2004) found that inhibition of the PI3-K pathway but not MAPK, showed insulin-forskolin driven CYP17 expression was decreased in non-PCOS theca cultures [45]. This suggests a focal point for examination within the insulin-signalling pathway where dysfunction may lie in PCOS theca cells.

7.2 Objectives

i. To culture primary human theca cells capable of survival through passage for later use in expression studies.

ii. To examine the expression of steroidogenic enzymes including CYP17 in non-PCOS and PCOS theca cells

iii. To examine the influence of hormonal treatments including LH and insulin, on CYP17 expression within non-PCOS and PCOS theca cell cultures.

iv. To examine the influence of LH and insulin on androstenedione secretion by non-PCOS and PCOS theca cells.

7.3 Methods

Ovarian specimens were collected based on specific patient criteria and following informed, written consent (*see 3.1*). Only small antral follicles <5mm in diameter were used for the study. Such individual follicles were hemisected. The theca interna, observed were micro-dissected with sharp forceps and enzymatically dispersed used to prepare for cell culture based on published methods Munir *et al.*, (2004) Mcallister *et al.*, (1994) Gilling Smith *et al.*, (1997) and described within section *3.2.3.* The isolated theca cells were then grown to confluence and passaged as required. Theca cell cultures were then incubated for 3-days in the absence of any additions or in the presence of insulin, LH, insulin + LH, insulin + PI3-kinase inhibitor (LY294002) or insulin + LH + PI3-kinase inhibitor for the aims stated above (*see 3.3*). Hormone and drug concentrations for the aforementioned were determined based on findings by Gilling-Smith *et al.*, 1994, Munir *et al.*, 2004, and Zhang 2000 who showed optimal ranges for activation and stimulation [45, 70, 192, 221]. All conditions used were shown to be supportive of the primary cultures and checked using proliferation assay, which also allowed examination of hormonal influence on proliferation. Protein expressions of CYP17, phospho-PKB and levels of androstenedione (see Chapter 8) were then compared between treated and untreated cells using western blotting, immunofluorescence and ELISA allowing us to examine the role of the insulin signalling pathway and its involvement in androgen synthesis within theca. Data were analysed as described in section *3.0.*

7.4 Results

7.4.1 Characterisation of non-PCOS and PCOS human theca cells in primary culture

It is well documented that primary human thecal cultures are difficult to establish and maintain in a differentiated functional state capable of steroidogenic activity [350-352]. This is thought to be due specific environmental/hormonal requirements difficult to pinpoint and reproduce *in vitro* and often requiring high serum supplementation that can affect treatment experiments. Even more difficulties are apparent in isolation of the specific theca cell types due to their location embedded deep within ovarian tissue and existing in close association with granulosa cells, typically functioning as a two-cell model.

Methods for theca cell isolation have been developed utilising intricate dissection protocols, which unfortunately yield limited cell numbers with reduced proliferative and metabolic ability [350]. During this project, in initial experiments and with a limited number of ovarian biopsies, it proved extremely difficult to perfect a suitable dissection technique and subsequent culturing protocol capable of sustaining a viable thecal primary culture. Using bovine ovaries kindly supplied by Dr Sinclair (School of Veterinary Medicine and Science, Sutton Bonnington, The University of Nottingham), we were able to hone the technique necessary to dissect individual follicles, and also experiment with the necessary media required to sustain the cultures (*see 3.2.3*). Although the bovine tissue varied significantly, particularly in the size of the follicles, we were able to transfer the technique to human cells and successfully maintain primary cultures to a passage of 7-8. With such difficulties with the protocol and reported frequency of granulosa contamination [350] it was necessary to carefully characterize the theca cultures. This was achieved by firstly examining theca cultures using immunofluorescence.

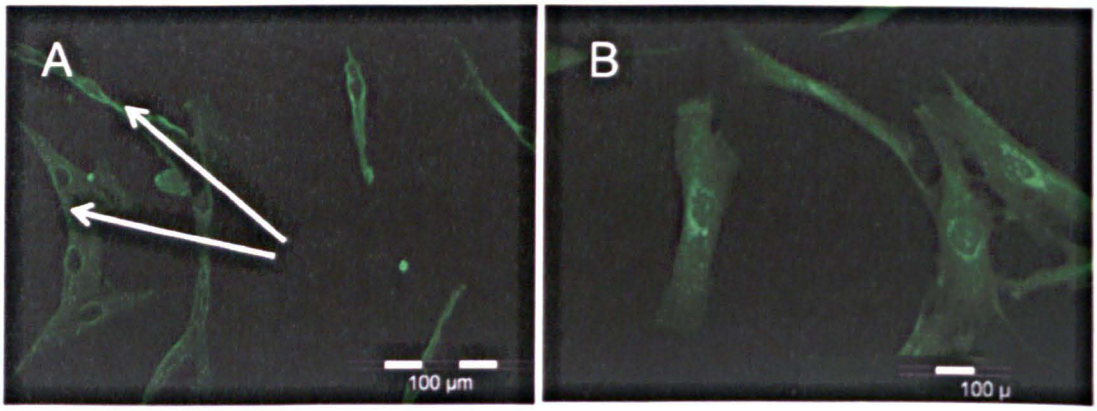

Figure 112. (A) Human theca cells positive control showing LHR expression. Isolated theca cells were probed with anti-LHR and FITC conjugated secondary antibody through immunofluorescence. Fluorescence representative of LHR localised to the membrane. **(B)** Immunofluorescence positive control for human theca cells showing β-Actin expression.

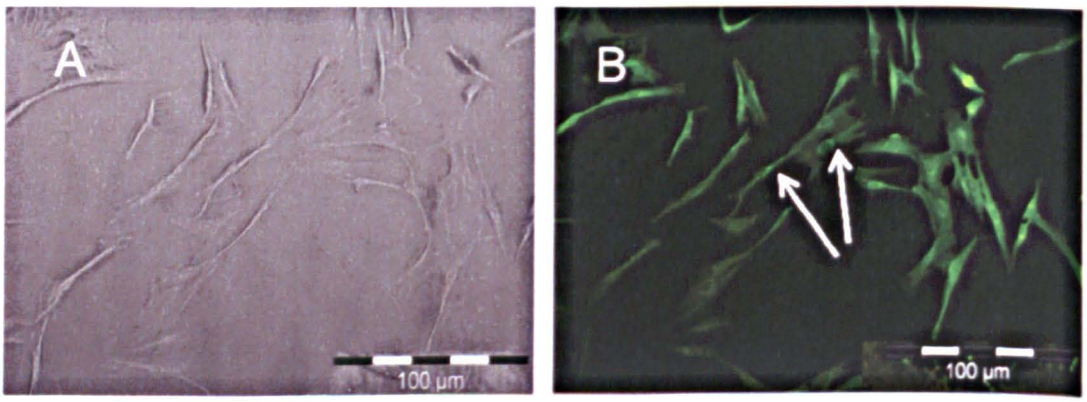

Figure 113. (A) Human theca cells at Day 5 passage 2, seen under bright field, showed elongated theca cell morphology. (B) Human theca cells showing CYP17 expression. Isolated theca cells were probed with anti-CYP17 and FITC conjugated secondary antibody. Fluorescence was also seen within the cytoplasm showing CYP17 with strong peri-nuclear localisation.

Theca cell cultures were grown to around 30-40% confluence to allow individual cell morphology to be seen (**Figure 113**). (A) Examination of the bright phase image shows the elongated shape as previously reported for theca cells *in vitro* [353].

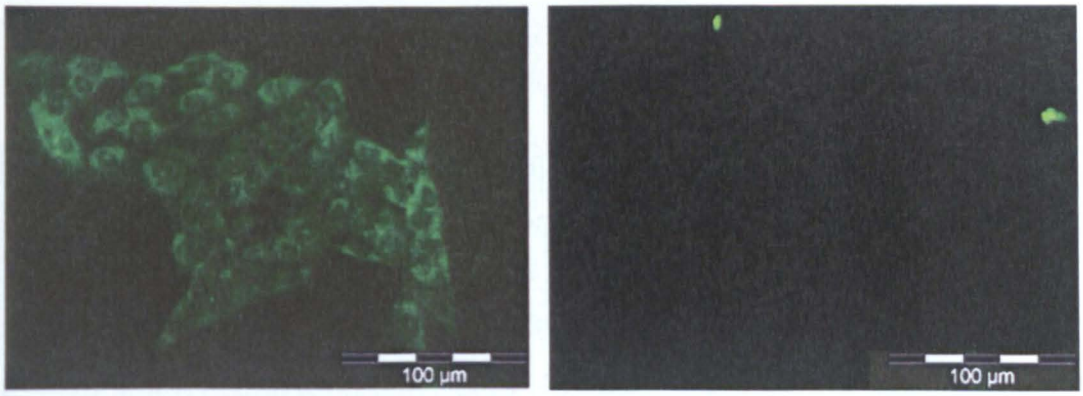

Figure 114. Chinese Hamster Ovarian cells showing CYP17 expression. Immunofluorescence of Chinese hamster ovary (CHO) cells showing CYP17 expression shows strong peri-nuclear fluorescence. CHO cells were used as a positive control for CYP17. Negative control also shown with no fluorescence seen.

From **Figure 113** we can see fluorescence representative of CYP17 expression localized to the cytoplasm. Peri-nuclear staining was also observed, suggestive of CYP17 synthesis. Although examination for CYP17 expression is a suitable marker for determination of theca cells [45] we decided to include further controls to fully support the primary cultures.

Luteinising hormone receptor antibody (LHR), which is shown to exist in theca cells (*see* **Figure 112**) but not present in granulosa [354], allowed fluorescence to be seen with in the cultures examined. Localisation was also seen within the cell membrane as reported with the presence of LHR [311]. Additional positive controls tested the protocol and CYP17 antibody. **Figure 114** shows Chinese hamster ovary cells (CHO), which have been shown to express CYP17. These were used as an alternate cell line and grown alongside the theca cells as a positive control [192, 279]. β-actin antibody was also used on all cultures examined to support the protocol.

Negative controls were used on all cultures and kept to the same exposure. Results showed lack of fluorescence (*see 3.5* and **Figure 114**). Leukocyte cultures as used by Wang *et al.,* (2005) (*see 3.5*) with no recorded presence of CYP17 expression or steroidogenic activity were cultured and probed with CYP17 antibodies [280]. Using secondary antibody alone and omitting the primary was also used to rule out non-specific binding. Finally cultured cells were grown and examined for CYP17

expression. No fluorescence was seen under examination showing no CYP17 presence.

In order to further support characterisation of theca cells and compare morphology of non-PCOS and PCOS cultures, both non-POCS (n=6) and PCOS (n=3) primary human theca cultures were stained with oil red O (*see 3.2.2.1*) for lipid droplet accumulation. When compared the non-PCOS cells, PCOS cells were observed to have greater numbers of lipids within the cytoplasm and large accumulations were found to clump in areas **Figure 115** (B).

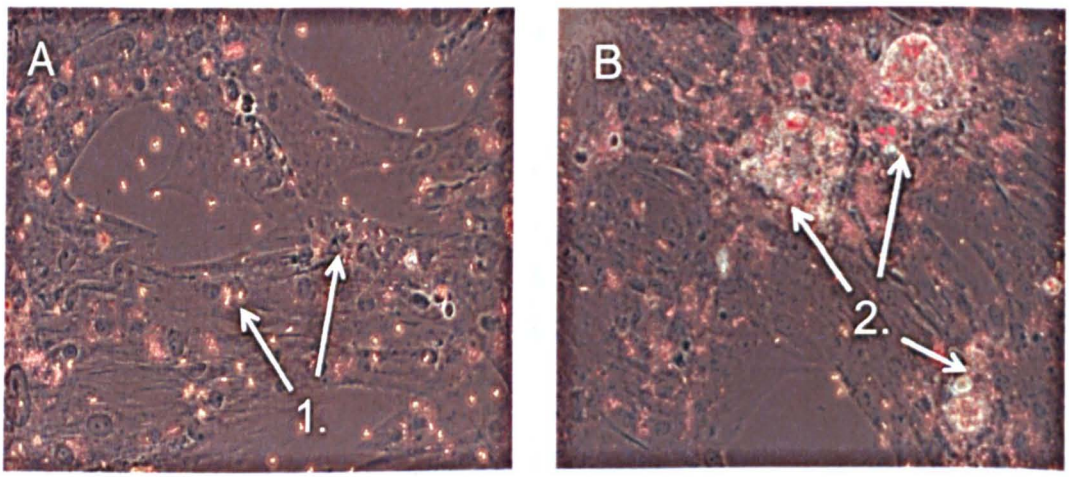

Figure 115. Human theca culture showing lipid droplet staining. (A) Non-PCOS theca cultures (n=6) were grown for 21 days and stained with oil red O to examine lipid droplet content (1). (B) PCOS human theca stained with oil red O. PCOS theca cell cultures (n=3) were grown alongside non-PCOS cells and stained with oil red O content to compare lipid droplet formation. **(2)** shows large lipid droplet accumulation and based on observations, a greater number of lipid droplets seemed present in PCOS theca cells over this period.

Following the determination of the theca cell presence and isolation (**Figure 113**, **Figure 115**) the steroidogenic pathway was examined. We focused on CYP17 due to its involvement in androgen synthesis through insulin signalling. Primary human non-PCOS (n=3) and PCOS (n=2) cultures were examined using immunofluorescence to determine whether any suggestion of variations existed in CYP17 expression between groups and therefore warrant further examination. Exposure time was reduced to 0.3s in both cell types due to the increased fluorescence seen in the PCOS cells. PCOS cells were observed to have increased fluorescence when compared to non-PCOS cells. This was suggestive of increased CYP17 expression and although this result

was not sufficient as a stand-alone quantitation, it allowed information supportive of further study using a more conclusive method of examination.

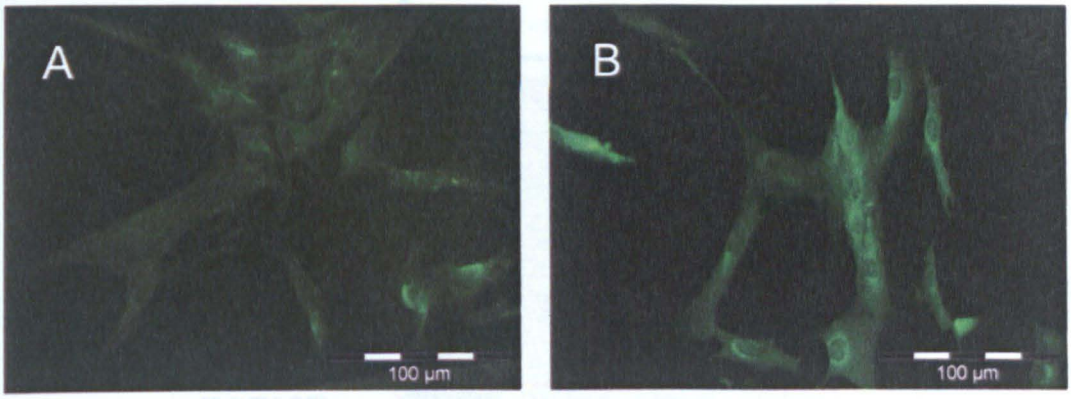

Figure 116. (A) Non-PCOS human theca immunofluorescence showing CYP17. Non-PCOS cultures (n=6) were probed for CYP17 expression using immunofluorescence and compared to PCOS thecal culture **(B)** (n=3). Exposure was kept to 0.3 seconds with fluorescence seen to be greater in PCOS cultures.

Western blotting was undertaken to confirm findings of CYP17 expression in lysates prepared from theca cultures of non-PCOS and PCOS. These were examined for CYP17 expression (n=4 vs. n=4) and protein bands ~57 kDa in size were observed in both groups. This was suggestive of CYP17 presence based on the predicted size of CYP17. Results seen in **Figure 117** and **Figure 118** show increased band intensity within the PCOS lysates when probed with CYP17 antibody but not within the non-PCOS group suggestive of increased expression. Quantification against β-Actin controls allowed for statistical analysis. Results showed a significant increase in PCOS band exposure against non-PCOS (see**Figure 118**). Western blotting was also used to examine non-PCOS and PCOS tissue homogenates. Unfortunately examination was limited to one sample due to availability (not shown). Optimisation required use of the majority of the PCOS theca lysate and we were unable to repeat this experiment to achieve a result suitable for quantification.

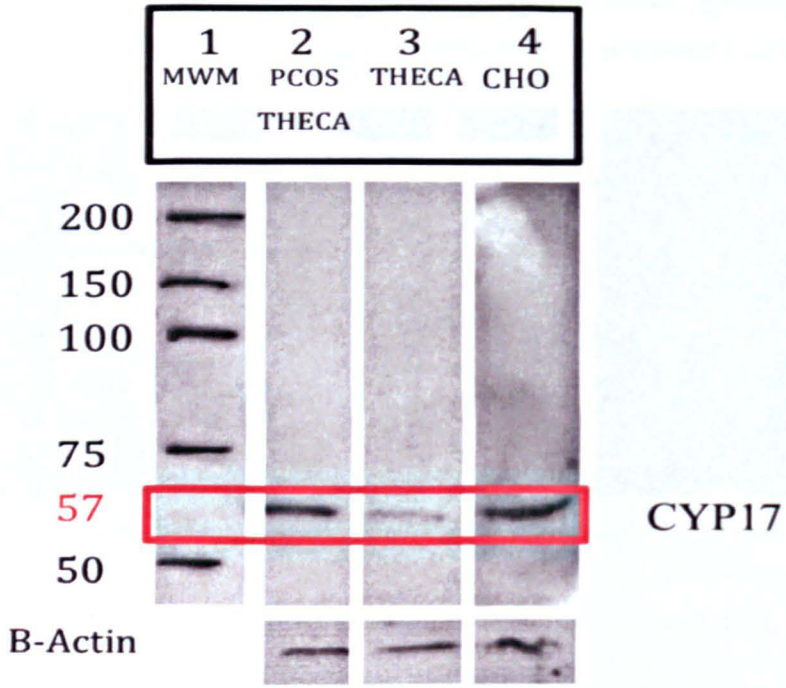

Figure 117. (A) Western blot analysis of CYP17 expression in human theca cells from non-PCOS and PCOS lysates (lane 3-4). Non-PCOS (n=4) and PCOS cultures (n=4) were examined for CYP17 expression. An immuno-reactive band was seen at 57kDa, in all lanes including the CHO positive control (lane 4) indicating CYP17 expression in these cells. Variation in band intensity was seen with PCOS showing results suggestive of greater expression in CYP17. β-Actin was used as a loading/positive control.

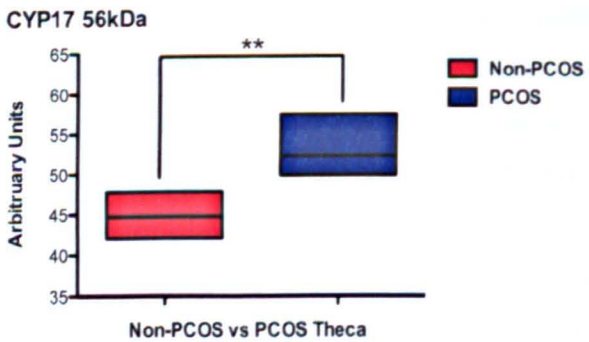

Figure 118. Summarising the densitometry representative of average expression of CYP17 in non-PCOS *vs.* PCOS theca lysates. Using β-Actin normalisation to compare levels of band intensity in western blot, CYP17 expression as shown in **Figure 117** densitometry graphs were created. Results showed significant difference were seen in expression of CYP17 in PCOS theca lysates (**P<0.01 showing means / ± SEM) using unpaired *t-test* statistical examination.

7.4.2 Proliferation in non-PCOS and PCOS theca

With difficulties surrounding establishing and maintaining primary human theca cell cultures it was necessary to ensure our conditions were suitable for proliferation. A growth curve of cells was first established in both non-PCOS and PCOS cultures. This allowed us to support the stability of our primary cultures. MTS assays were used to colourmetrically assess proliferation and cell viability (*see 3.4*). Although examination of theca cell proliferation from non-PCOS and PCOS subjects showed an increased trend in PCOS theca cell proliferation, sample numbers were not sufficient for statistical significant.

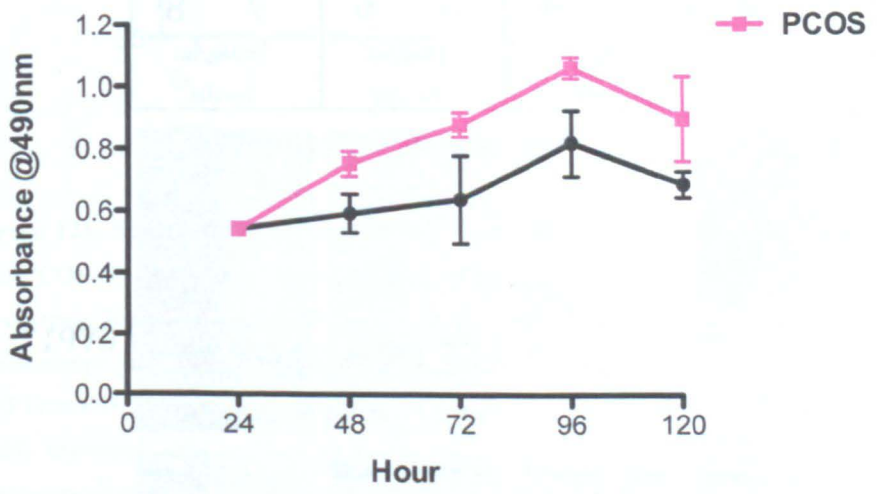

Figure 119. Non-PCOS and PCOS theca cell proliferation. Non-PCOS and PCOS cell cultures (N=3 vs. 2) were grown for 5 days and proliferation examined using MTS assay. Results are mean ± SEM. Proliferation was seen with no significance between non-PCOS and PCOS cells. However a trend toward increased PCOS theca proliferation would support further examination through increased sample size.

7.4.3 The influence of insulin and LH on CYP17 expression in non-PCOS and PCOS theca cells

Thecal cell cultures were processed into primary cultures from biopsies taken from non-PCOS and PCOS patients and cultured to passage 3 to increase cellular yield (n=3) (*see 3.2.3*). Cultures were then treated with varying insulin treatments (0-100 ng/ml) for 3 days. The cells were then lysed and proteins extracted for use in western blot analysis. CYP17 antibody (1:50 Abgent AP7879c) was used to probe blots and comparisons of non-PCOS and PCOS CYP17 expression made.

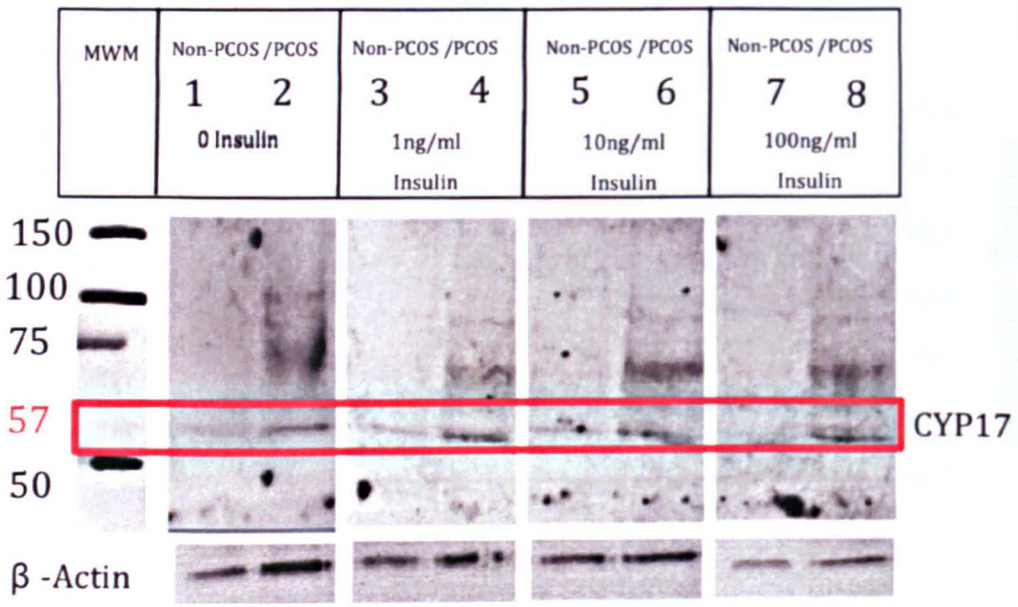

Figure 120. Western blot analysis of CYP17 expression in non-PCOS and PCOS human theca cells under varied insulin treatments. Human theca cultures (n=3) were treated to varied insulin doses (0-100ng/ml) and harvested for western blot analysis. CYP17 antibody probing showed no variation occurred under varied treatments in non-PCOS or PCOS groups.

Figure 120 shows an increase in CYP17 expression was seen between non-PCOS and PCOS groups throughout all treatment. **Figure 120** also showed that although insulin treatment was seen to show slight increase in expression between untreated and treated, no variation was shown with increase in insulin treatments in either group. Statistical analysis supported this observation and significant increases were seen in expression of CYP17 between PCOS and non-PCOS theca lysates throughout all insulin treatments (*$P<0.05$/**$P<0.01$). However no significant differences were seen

across the insulin treatments range in CYP17 expression within non-PCOS and PCOS groups see **Figure 121**.

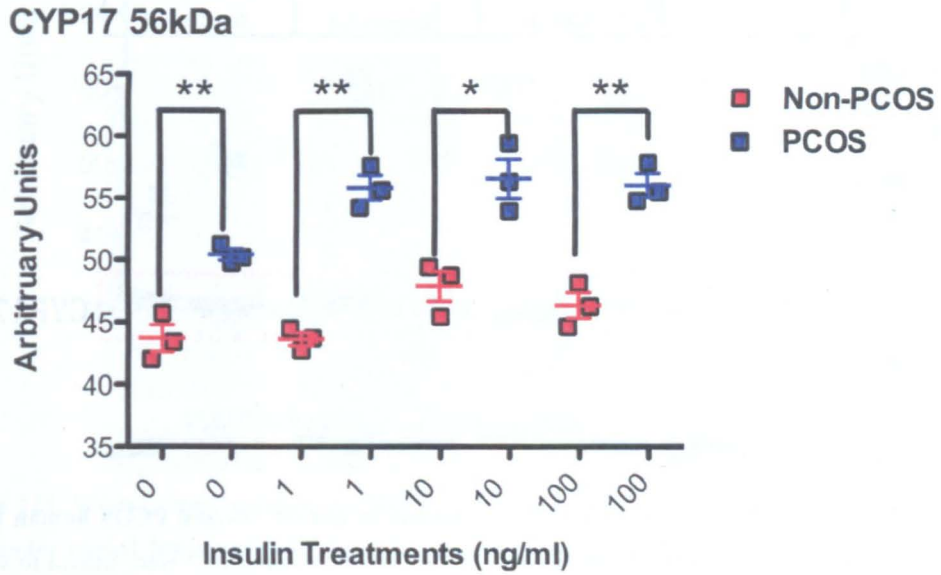

Figure 121. Summarising the densitometry representative of average expression of CYP17 in non-PCOS *vs.* PCOS theca lysates under varying insulin treatments. Using β-Actin normalisation to compare levels of band intensity in western blot CYP17 expression as shown in **Figure 120** densitometry graphs were created. Results showed significant increases were seen in expression of CYP17 in PCOS theca lysates throughout all insulin treatments (*P<0.05/**P<0.01 showing means / ± SEM) examined using oneway ANOVA. No significant differences were seen across the insulin treatments range in CYP17 expression within non-PCOS and PCOS groups.

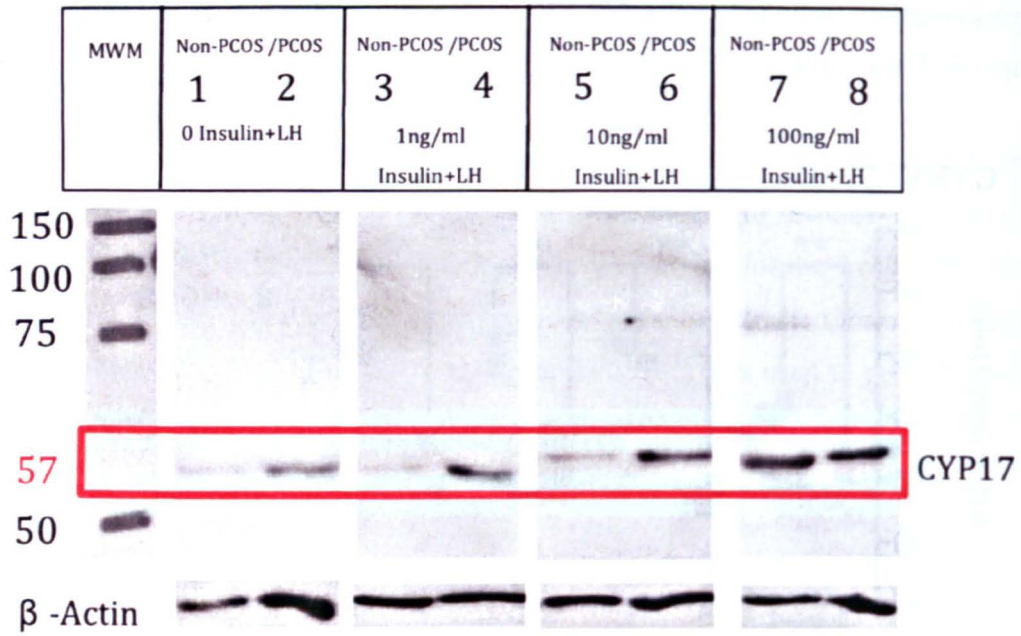

Figure 122. Western blot analysis of CYP17 expression in non-PCOS and PCOS human theca cells under varied insulin and LH treatments. Human theca cultures (n=3) were treated to varied insulin doses (0-100 ng/ml) with and without LH (10ng/ml) and harvested for western blot analysis. CYP17 antibody probing showed variation occurred with increased CYP17 expression seen with the increase in insulin and the addition of LH treatments.

Following examination of the effects of insulin treatment alone had on non-PCOS and PCOS groups, I examined the effects using the same insulin range with the addition of LH (10ng/ml) as recommended by Gilling-Smith *et al.*, (1994) to allow for the optimum response when examined as an individual treatment in thecal cultures [221]. Western blot was used to determine CYP17 expression. Similarly to insulin treatments alone, increased CYP17 expression was seen between non-PCOS and PCOS groups throughout all treatments and supported statistically when examined against β-Actin normalization (*$P<0.05$). Furthermore, CYP17 expression was also seen to increase in both non-PCOS and PCOS with the addition of LH treatment, with the largest expression seen following 100ng/ml insulin + 10ng/ml LH in both groups **Figure 122**. See *3.0* for further details.

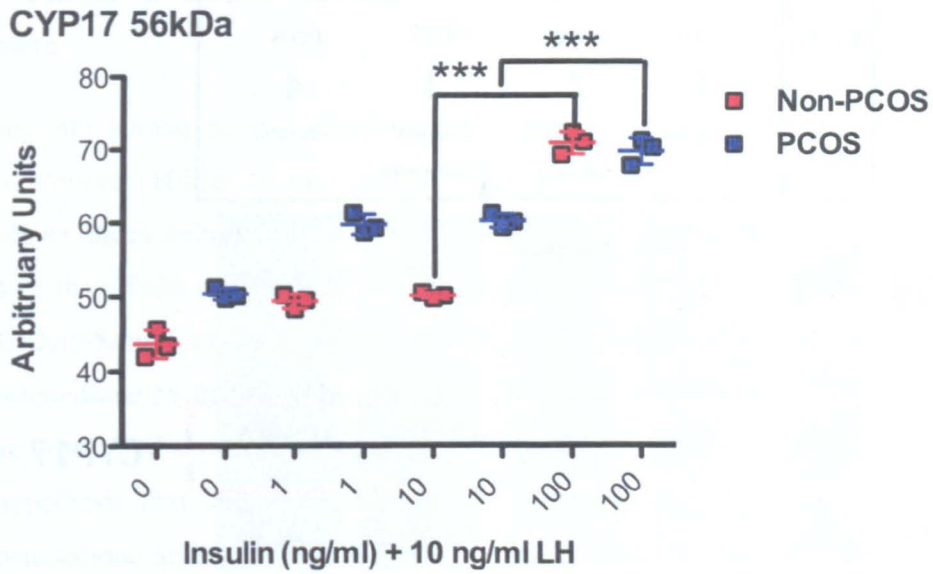

Figure 123. Western blot analysis of CYP17 expression in non-PCOS and PCOS human theca cells under varied insulin and LH treatments. Using β-Actin normalisation to compare levels of band intensity in western blot, CYP17 expression as shown in **Figure 122** densitometry graphs were created. Results showed significant increases were seen in expression of CYP17 in PCOS theca lysates throughout all insulin treatments and LH treatments (Sig not shown *P<0.05). Significance was also seen within non-PCOS and PCOS groups with increases in insulin treatment (Sig not shown *P<0.05) with the largest increase seen from 10-100 ng/ml insulin +10ng/ml LH (***P<0.001 means / ± SEM) as shown here following oneway ANOVA analysis.

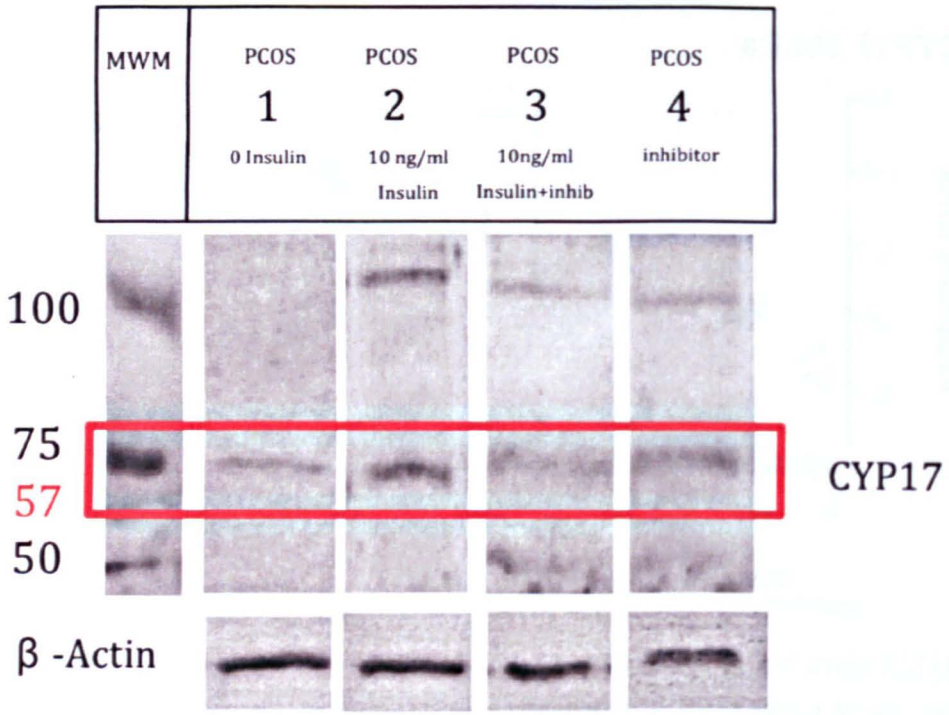

Figure 124. Western blot analysis of CYP17 expression in PCOS human theca cells under insulin and PI3-K inhibition. Human PCOS theca cultures (n=2) were stimulated using 10ng/ml insulin (lane 1) with and without the addition of the PI3-K inhibitor LY294002 and examined for CYP17 expression using western blot technique. Results showed that inhibition of PI3-K showed noticeable decreases in CYP17 expression when compared to insulin alone.

Using the western blot technique we began to examine the involvement of the PI3-K pathway through activation using insulin treatment as shown by Munir *et al.*, (2004) and the levels of CYP17 expression [45]. Inhibition of the PI3-K pathway was achieved through incubation of the theca cultures with LY24002 (*see 3.3*). This was followed by the addition of insulin treatment known to stimulate the PI3-K pathway [45]. Lysates were created from the cultures (n=2) and examined for CYP17 expression. **Figure 124** shows no variation in CYP17 expression to the application of either 0 or on using LY294002 inhibitor alone. A combination of insulin and PI3-K inhibitor however suggested reduction in CYP17 expression. Further results are required to substantiate this.

7.4.4 Effects of insulin on non-PCOS and PCOS Theca androstenedione secretion

Studies into human ovarian steroidogenesis have typically been limited to ovarian slices however Hillier *et al.*, (1991) were one of the first groups to establish monolayer theca cultures in serum free media [355]. From this they were able to examine the effects of activin A on androgen inhibition. This method was also used by Gilling-Smith *et al.*, (1994) in the examination of hyper secretion of androstenedione by PCOS theca cells [91]. They were able to examine the effects of LH treatment and showed increases over basal androstenedione secretion [91]. With the hypothesis that augmented insulin and LH leads to even greater increase in androstenedione secretion both physiologically and *in vitro,* and the understanding that hyper secretion of these hormones occurs within PCOS patients we examined the effects of LH and insulin treatments. This was done within a serum free monolayer theca culture processed from biopsies taken from non-PCOS and PCOS patients (*see 3.3.2*).

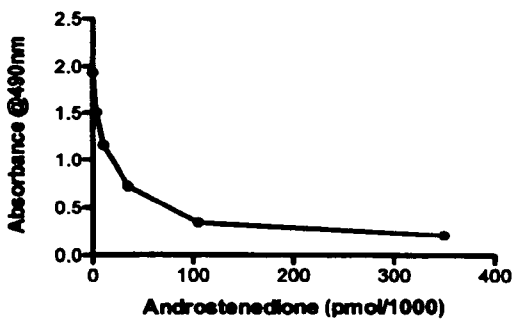

Figure 125. Standard curve showing ELISA androstenedione levels through measured absorbance. Known levels of androstenedione were measured using ELISA technique and levels of absorbance @490 taken to determine a standard curve.

Using the androstenedione ELISA we measured known levels of androstenedione to create a standard curve. Unknown values were then interpolated to determine androstenedione levels in our samples, (*see 3.7*) see **Figure 125**. This also ensured that androstenedione levels measured in our cultures fell into the range capable of being read by our chosen ELISA (0.0019ng/ml / 0.03pmol/1000 cells).

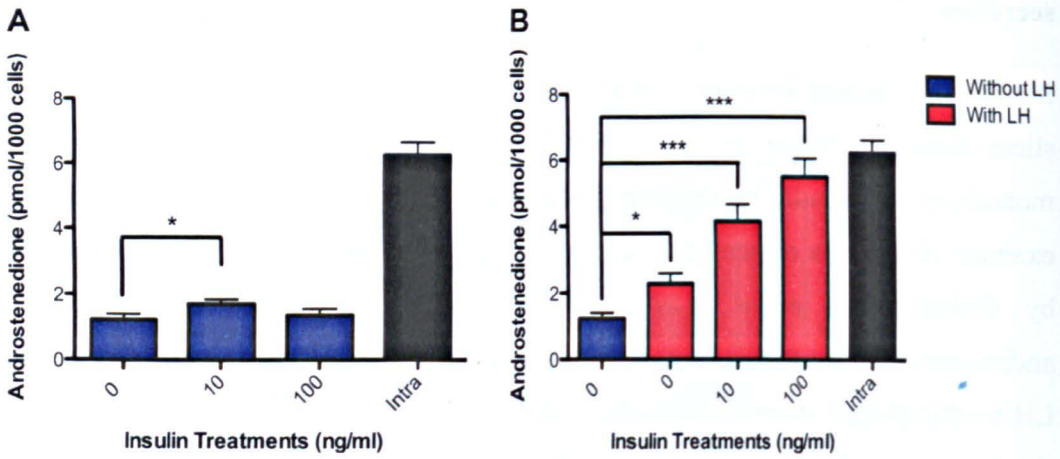

Figure 126. (A) Androstenedione secretion from non-PCOS human theca under varied insulin treatments. Human theca cells were cultured (n=9) and treated with varying doses of insulin (0-100ng/ml) for 3 days. Results are mean ± SEM. Media was harvested and measured for androstenedione using ELISA technique. Results showed a small but significant increase in androstenedione secretion was observed following 10ng/ml insulin treatment (*P<0.05) with no further difference seen across treatment ranges. Data analysed using oneway ANOVA **(B)** The addition of LH (10ng/ml) to each insulin treatment was seen to increase androstenedione presence. A significant increase in androstenedione secretion was observed following LH treatment. Insulin treatment showed significant increases in androstenedione secretion in a dose dependent manner when treated with a single dose of LH (10ng/ml) (***P<0.001) and 100ng/ml insulin +LH (***P<0.001). Data analysed using oneway ANOVA.

Once established human theca cells from non-PCOS and PCOS biopsies were cultured to passage 3 to increase cell yield, $10x10^4$ cells were then plated in serum free media and treated with varying doses of insulin (0-100ng/ml with and without LH (10ng/ml) for 3 days. Within 24 hours the cells had adhered and formed colonies and by 48 hours they showed cell morphology in accordance to published results with elongated spindle shaped appearance [350]. This was seen throughout all treatments. After 72 hours media was harvested and examined for androstenedione using ELISA technique (*see 3.3*). Examination of non-PCOS harvests (n=9) showed that a small but significant increase in androstenedione secretion was observed following 10ng/ml insulin treatment (*P<0.05). However, 100ng/ml insulin treatment shows no difference in androstenedione secretion from basal (**Figure 126**). However the addition of 10ng/ml LH to each insulin treatment showed significant increases with a

1.8 fold increase over the basal level with LH alone (median basal 1.3pmol/1000 cells, 0 insulin +LH=2.4pmol/1000 cells), a 3-fold increase seen with the addition of 10ng/ml insulin +LH over the basal, and 4.3-fold increase over the basal with 100ng/ml insulin +LH (**Table 8.**).

	0 Insulin	0 Insulin + LH	10ng/m Insulin +LH	100ng/ml Insulin +LH	Intra
Minimum	0.48	1.08	1.67	2.16	5.48
25% Percentile	0.71	1.37	3.10	5.01	5.48
Median	1.31	2.40	4.00	5.71	6.50
75% Percentile	1.70	2.79	5.55	6.33	6.71
Maximum	1.78	3.70	6.08	7.50	6.71
Mean	1.22	2.28	4.14	5.49	6.23
Std. Deviation	0.48	0.87	1.46	1.55	0.65
Std. Error	0.17	0.30	0.51	0.55	0.37

Table 8. Table of variation in androstenedione across insulin and LH treatments in non-PCOS theca cells. Androstenedione secretion was examined under insulin +/- LH treatments. Statistics showed 1.8 fold increase over the basal level with LH alone; a 3 fold increase seen with the addition of 10ng/ml insulin +LH over the basal; and 4.3 fold increase over the basal 100ng/ml insulin +LH.

Further comparison of insulin treatments against insulin and LH, within non-PCOS **Figure 127** showed significant increases in androstenedione under insulin and LH treatments, across all groups with the largest increases seen in 100ng/ml and LH and showing a positive correlation with increased insulin. These results suggest that insulin and LH act to augment and increase androstenedione secretion in human theca cells and that this effect is not present with insulin alone. It also shows that LH alone also has a positive effect on androstenedione secretion.

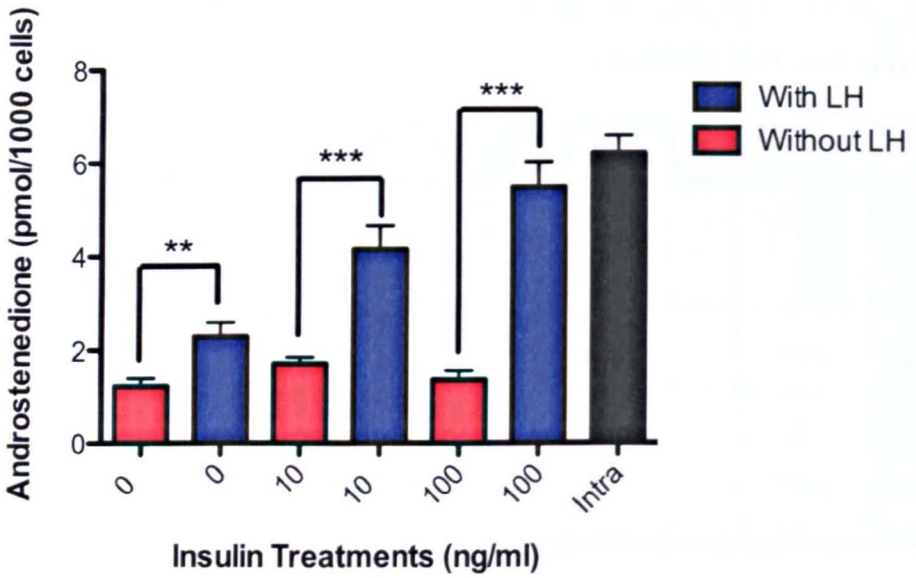

Figure 127. Comparison of androstenedione secretion in non-PCOS human theca following 3 day treatments with varying insulin +/-LH. Human theca media harvest (n=9) were taken following 3 day treatments with varying insulin (0-100ng/ml) +/- LH (10ng/ml). Results are mean ± SEM. Comparison of insulin treatments with and without LH showed significant increases in androstenedione in all insulin treatments with the addition of LH. A dose dependent increase was seen with increased insulin treatments in all LH groups (0 insulin +LH **P<0.01) (10-100 ng/ml insulin +LH ***P<0.001). Data analysed using oneway ANOVA following *bonferroni* post-hoc test.

Figure 128 showed no variation in androstenedione under varied insulin treatments within PCOS theca media harvests. However comparison of non-PCOS with PCOS (**Figure 130 (A)**) showed significant variation in basal levels (***P<0.001) suggesting increased androstenedione over non-PCOS secretion by PCOS theca without any hormonal influence.

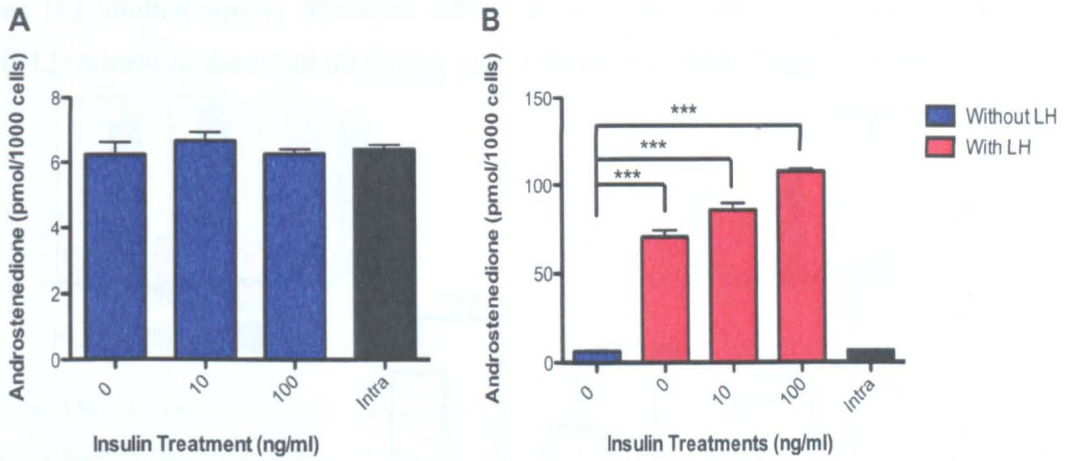

Figure 128. (A) Androstenedione secretion from PCOS human theca under varied insulin treatments. Human theca cells were cultured (n=4) and treated with varying doses of insulin (0-100ng/ml) for 3 days. Media was harvested and measured for androstenedione using ELISA technique. Results are mean ± SEM. Results showed androstenedione was present with no significant variation occurring across treatment ranges. **(B)** The addition of LH (10ng/ml) to each insulin treatment was seen to increase androstenedione levels. A significant increase in androstenedione secretion was observed following LH treatment. Insulin treatment showed significant increases in androstenedione secretion in a dose dependent manner when treated with a single dose of LH (10ng/ml) (***P<0.001) and 100ng/ml insulin +LH (***P<0.001). Data analysed using oneway ANOVA following *bonferroni* post-hoc test.

	0 Insulin	0 Insulin + LH	10ng/m Insulin +LH	100ng/ml Insulin +LH	Intra
Minimum	5.07	63.00	80.40	104.00	6.25
25% Percentile	5.37	64.00	80.75	105.00	6.25
Median	6.51	69.85	82.65	109.00	6.39
75% Percentile	6.82	77.95	93.63	110.00	6.53
Maximum	6.85	79.7	97.00	110.00	6.53
Mean	6.23	70.6	85.68	108.00	6.39
Std. Deviation	0.81	7.256	7.65	2.82	0.19
Std. Error	0.40	3.628	3.82	1.41	0.14

Table 9. Table of variation in androstenedione across insulin and LH treatments in PCOS theca cells. Androstenedione secretion was examined under insulin +/- LH treatments. Statistics showed 10 fold increase over the basal level with LH alone; a 12.6 fold increase seen with the addition of 10ng/ml insulin +LH over the basal; and 16.1 fold increase over the basal 100ng/ml insulin +LH.

Significant rises were also seen between insulin treatment groups without LH and insulin with LH (**Figure 129**) with further rises seen with increases in insulin+LH in all PCOS cultures.

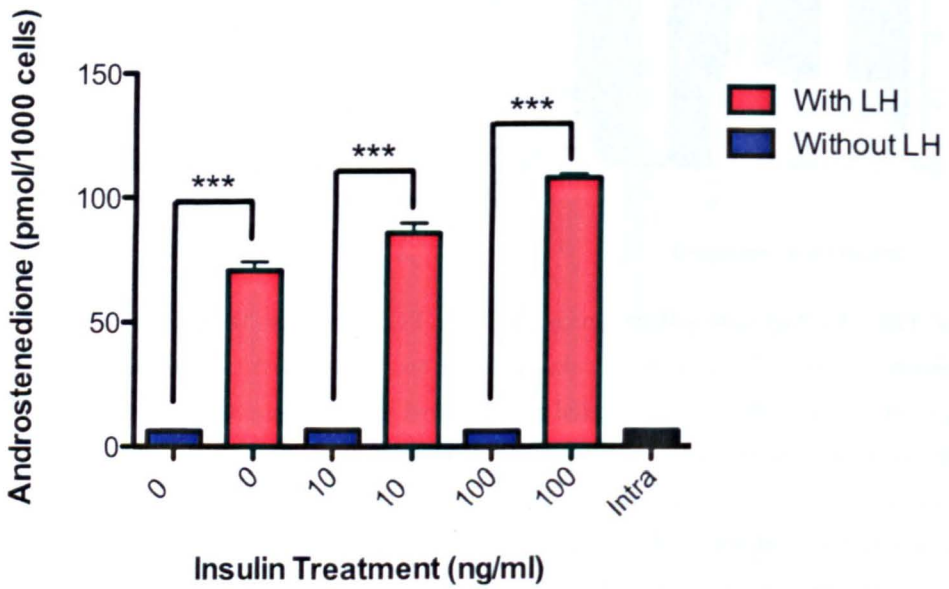

Figure 129. Comparison of androstenedione secretion in PCOS human theca following 3 day treatments with varying insulin +/-LH. Human theca media harvest (n=4) were taken following 3 day treatments with varying insulin (0-100ng/ml) +/- LH (10ng/ml). Results are mean ± SEM. Comparison of insulin treatments with and without LH showed significant increases in androstenedione in all insulin treatments with the addition of LH. Positive correlation was seen in increased insulin treatments in all LH groups (0 insulin +LH ***P<0.001) (10-100 ng/ml insulin +LH ***P<0.001). Data analysed using oneway ANOVA following *bonferroni* post-hoc test.

Furthermore the addition of LH to increasing insulin treatments had large effects on the androstenedione levels measured with levels increasing ~tenfold from 6.5pmol/1000 cells basal to 69.8pmol/1000cells with the addition of LH (10ng/ml). Increasing the insulin treatment with the addition of LH showed further increases with a 12.6-fold increase seen with 10ng/ml insulin and a 16.1-fold increase seen in 100ng/ml insulin (**Table 9**). Comparison of non-PCOS with PCOS androstenedione levels under insulin treatment with the addition of LH showed significant increases in androstenedione within groups shown in **Figure 130** B (***P<0.001).

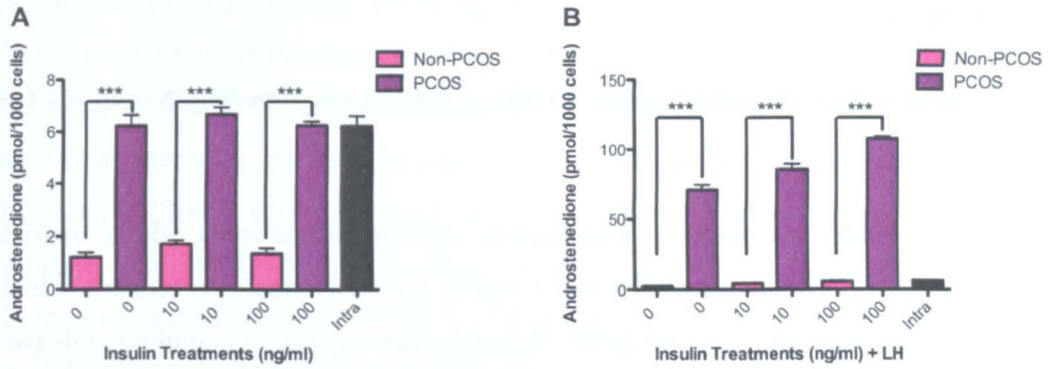

Figure 130. (A) Androstenedione secretion under varying insulin treatments from non-PCOS and PCOS human theca cultures. Following 3 day varied insulin treatments of non-PCOS (n=9) and PCOS (n=4) theca cultures androstenedione levels were measured in media. Results are mean ± SEM. Significant variation in androstenedione secretion was seen with insulin treatments between non-PCOS and PCOS levels (***P<0.001). (B) Androstenedione levels were also measured from cultures treated with varying insulin with the addition of LH (10ng/ml). A significant increase in androstenedione secretion was seen in a dose dependent manner when treated with a single dose of LH (10ng/ml) within all treatment groups between non-PCOS and PCOS (***P<0.001). Data analysed using oneway ANOVA following *bonferroni* post-hoc test.

7.5 Discussion

7.5.1 Culture and characterisation of theca cells from non-PCOS and PCOS women

Thecal steroidogenesis works in conjunction with granulosa cells within ovarian tissue as a two-cell model whereby theca steroid synthesis creates precursors which are transported, trans-membranously for further processing within granulosa cells (*see 1.4.1*) [356]. The morphology of the follicle tissue is such that separation and isolation of theca cells is notoriously difficult [350] due to size of the follicle, the availability, the tight association to surrounding tissue and the fragility of the tissue itself. In order to establish primary theca cultures and characterise them fully, it was necessary to thoroughly control for granulosa contamination. Any co-culturing would later affect levels of hormone secretion and subsequent enzyme activity and expression. CYP17 is involved in the androstenedione pathway not present in granulosa cells [9, 20]. This is also true of the LH receptor, which is present in theca cell regulation and metabolism [357]. Immunofluorescence results showed positive findings of both these markers supporting theca cell phenotype. This was also seen in the western blot results with CYP17 presence seen in both non-PCOS and PCOS lysates. Separate culturing of granulosa cells allowed validation of the protocol and controls and showed no CYP17 or LHR presence (data not shown).

Other characteristic markers of theca cells further supported the establishment of a predominantly theca culture. The elongated cellular morphology and presence of lipids in both cultures further supported the existence of isolated theca cells [350, 358].

Theca cells contain structural features including plentiful mitochondria comprising vesicular cristae, as well as cytoplasmic granular endoplasmic reticulum, and lipid vesicles as shown by Magoffin (2005) [359]. The presence of lipid vesicles allowed us to examine lipid droplet accumulation through oil red O staining as well as examining variations that may exist between non-PCOS and PCOS cultures. With the hypothesis that PCOS thecal cells may contain a dysfunction associated with increased steroid synthesis [360], analysis of lipid droplet accumulation was of interest as they may give evidence for increased hormonal synthesis in PCOS theca and it has been reported that the lipid droplets are used as cellular storage containing

the precursors required for steroidogenesis [358]. Although not quantified the observations showing increased numbers of lipid droplets in our PCOS results when compared with non-PCOS cultures, may therefore be explained through the increased demand for steroid synthesis in PCOS cells. This is supported by research by Convery and Brewer (2005) who examined thecal rat cells and found increased large and clumped lipid cells in experimentally induced PCO rats [154]. Although this observation is made in human cells, the similar occurrence gives further evidence to a possible variation in PCOS and rat models are becoming more frequently used to examine the condition as they exhibit both the metabolic and morphological characteristics of human PCOS [361]. Although this is an excellent model for experimentation, physiological variations in human and rat samples emphasise the importance of results taken from working with human samples. It should also be noted that although reduced, granulosa have been reported to exhibit lipid like granular components and this characterization alone would not be enough to support identification of theca cells but was intended more as a comparative examination across groups. This method was also not quantifiable and purely observational. We could adapt this method through removal of the oil red O stain and measurement of absorbance. Comparison against controls could show variations in stain indicating variation in lipid content. Alternatively, measurement with quantitative reverse transcription-PCR analysis of lipid metabolism-related genes, such as carnitine palmitoyltransferase (CPT) I, fatty acid binding protein, pyruvate dehydrogenase kinase-4, and NADP-dependent cytosolic malic enzyme, would allow a more in depth analysis as described by Kondo *et al.*, (2005) [362].

PCOS has been associated with thecal hyperplasia and ovarian enlargement both of which have been hypothesised to be linked with increased androgen synthesis [266, 363]. Our results, although showing a trend to increased proliferation within PCOS theca, showed no significance between groups. This may be attributed to limited sample groups. However Givens (1984) examined PCOS patients with associated ovarian follicular cysts that showed no signs of theca hyperplasia [364]. Further examinations into the area suggested classification was necessary with type IV PCOS showing reduced follicular cysts but with noticeable hyperplasia and 'hyperthecoesis' [364, 365]. This would later support the Rotterdam classification of PCOS (Rotterdam, 2004) where endocrinological features were noted in the absence of morphological

features of PCOS. Limitations in sample collection may explain variations seen within our results. This may go someway to explain why our findings go against existing data that suggests thecal hyperplasia in PCOS [102, 366]. The results do however show that the growth environment was suitable for proliferation.

7.5.2 The influence of insulin and LH on CYP17 expression in non-PCOS and PCOS theca cells

Research has shown that insulin acting through ovarian receptors and in combination with LH, allows stimulation of androgen synthesis [42, 161]. This supports the hypothesis that symptomatic hyperinsulinemia and hyper LH secretion in PCOS plays an important role in the aetiology of ovarian linked hyperandrogenism. Further experiments have examined the use of metformin and troglitazone, which are used as insulin sensitizing agents. La Marca *et al.*, (2000) showed that metformin treatment reduced stimulated ovarian CYP17 activity in hyperinsulinemic PCOS women [367], suggesting that insulin resistance found in PCOS patients may play a key role in hyperandrogenism. If this were to be considered true, further study into the exact defect within the insulin signalling pathway would require careful examination.

Studies have also shown that no variations exist in the function or expression of IRS-1/2 associated PI3-K in PCOS fibroblasts or adipocytes when compared to non-PCOS, it is therefore believed that alterations in signal regulation exist downstream of these proteins [368, 369]. Our examination concentrated on the insulin stimulated PI3-K pathway and our results support the treatment ranges as published by Munir *et al.*, (2004) with 10ng/ml insulin stimulating the pathway through its activation of IRS-1 and that the resultant effects of the specific inhibitor LY294002 [45], were seen to be effective in reducing the response. This result also supports the involvement of the PI3-K pathway. It was intended to further investigate this pathway by examining PKB phosphorylation associated with PI3-K activation. Although we carried out initial western blot probing for PKB and phosphorylated-PKB in all the lysates examined for CYP17 we were unable to support the finding with sufficient repetition. Our results from examining the inhibition of the PI3-K pathway in theca cells did however suggest a subsequent reduction in CYP17 expression. Further work would be required in order to found these results that included more repeats and PKB examination.

Our results show that insulin treatment alone did not increase CYP17 expression. This finding supports previous studies into thecal steroidogenic activity that show the same result [45]. Furthermore, our examination into PCOS cultures shows this to be true in PCOS theca cells *in vitro* and western blot results, showed increased band intensity within the PCOS group suggestive of increased CYP17 expression within these cells. In conclusion our results suggest that although there is no dysfunction within the effect of insulin on non-PCOS and PCOS theca cells on CYP17 expression, increased CYP17 expression exist within PCOS theca independently.

The co-stimulatory effect of LH and insulin on CYP17 expression shown in our results gives evidence to support that co-activation of the androgen synthesis pathway is required through both insulin and LH. Furthermore our results show CYP17 is reduced in comparison, within individual insulin or LH treatments. Munir postulated that this stimulation is different to that seen in glucose uptake, as it requires insulin alone. Insulin thecal androgen regulation through alternate intracellular mechanisms to that of glucose, seems feasible. This is therefore supported by the requirement of co-activation of the PI3-K and cAMP pathway [45]. If this is the case and with our results showing LH+Insulin increases in CYP17 expression in non-PCOS and PCOS theca cells, then a possible pathway divergence may exists downstream to PI3-K that would regulates androgen synthesis through insulin activation independently of glucose metabolism.

7.5.3 The influence of insulin and LH on androstenedione secretion in non-PCOS and PCOS theca cells

The objective was to determine the effects of insulin and LH both individually and combined, on androstenedione secretion in cultured theca cells. This was to be examined in both non-PCOS and PCOS cultures to allow comparison and to determine if any variation existed that may be associated to symptomatic hyperandrogenemia. Due to the effects serum may have on steroidogenesis through insulin supplementation a similar serum free monolayer culture was established as used by Gilling-Smith *et al.*, (1991) [91]. Examination of non-PCOS cells showed that insulin alone had no effect on androstenedione levels throughout the chosen treatment range (10-100ng/ml). Similar results were shown by Stewart *et al.*, (1995)

in examination of bovine theca cultures using identical treatment range and showing no variation in androstenedione secretion within cultures through radioimmunoassay techniques [161]. This has also been shown in rat interstitial theca cells when subjected to 100ng/ml insulin treatments leaving basal levels unaffected [162]. Magoffin and Weitsman (1994) have also shown this to be true for IGF-1 with an inability to stimulate steroidogenesis through its lack of cAMP or protein kinase A production [370]. Some contradictory studies do exist such as Barbieri *et al.*, (1984) who showed that examination of ovarian stroma of women with hyperandrogenemia showed androstenedione secretion when stimulated equally by an insulin treatment range 50-500ng/ml [156]. However these early studies can be criticised through use of direct tissue incubations that may lead to discrepancies from alternate cell contamination. These studies also used serum-supplemented media. Stewart *et al.*, (1994) also noted that these studies failed to account for cell numbers following incubatory periods, which would also affect measured basal secretory levels [161]. Based on this we can support findings that show the stimulatory effect of insulin on androstenedione secretion in *in vitro* theca cells is unaltered through increased insulin treatment within the range 10-100ng/ml.

Examination of insulin treatments across non-PCOS and PCOS cultures does however show a marked increase in androstenedione levels suggestive of increased basal levels secretion in PCOS cells (***$P<0.001$). This has been shown previously by Gilling-Smith *et al.*, (1994) who reported non-PCOS basal levels around 1.7pmol/1000 cells against basal PCOS androstenedione 32pmol/1000 cells [221]. Although this supports our findings the PCOS basal androstenedione levels reported in this study are higher than those found in our results [91, 128].

Interestingly our results show no variation between basal and insulin treated PCOS androstenedione secretion. Similar results were shown by Stewart *et al.*, (1995) when examining bovine theca, it should be noted that an outlying value does exist within our data that prevents significance [161]. Although there are few studies specifically focused on PCOS thecal androgen synthesis under insulin treatment, research by Zhang *et al.*, (2000) has shown that a treatment of 100ng/ml insulin showed a small increase over basal androstenedione secretion [371]. Furthermore Nestler *et al.*, (1996) also reported insulin stimulation of testosterone synthesis in PCOS theca

cultures [157]. Furthermore it has been shown that CYP17 is reduced in PCOS women following reduced insulin secretion [372]. Our result may however, be suggestive of active steroidogenesis in PCOS theca that leads to increased androgen secretion regardless of insulin stimulation. Examination into the influence of insulin action has shown variation in responses within different target tissues in samples taken from PCOS patients. These included fibroblasts, skeletal muscle and adipocytes. Although these studies focused on the mechanism of insulin regulation, including binding sites and receptor response and showed a 30% reduction in adipocyte auto-phosphorylation (Ciraldi, 1992). This may suggest that although stimulation is seen in non-PCOS theca cells, variation in PCOS theca response exists similar to other target tissue variation. The paradox has always existed as to how insulin resistant women found in PCOS have hyper responsive ovarian androgen synthesis as a result of compensatory increased hyperinsulinemia. With this in mind the answer maybe, this is not the case. Although the evidence fails to support this, it should not be completely ruled out and with the variations found and limited research into *in vitro* examination of PCOS theca cells, further examination into the influence of insulin action alone on androgen synthesis would be of interest. It may also be that insulin requires co-stimulation of androgen synthesis. This seems likely with the evidence of LH augmentation in both non-POCS and PCOS theca cells.

Examination of LH stimulation of both non-PCOS and PCOS theca cells showed increased levels of androstenedione over both insulin stimulated and basal levels as found in similar studies in *in vitro* bovine and rat theca. This was found to be true in both non-PCOS and PCOS groups. Further to this a significant increase was seen in the effect of LH treatment on androstenedione in comparison of non-PCOS and PCOS groups. This result was seen in research by Gilling-Smith et al., (1995) and although they determined an even greater response to LH by PCOS in androstenedione secretion, this may be accounted for through their examination of follicle size where they found that theca cells derived from follicles <10mm produced significantly less androstenedione than those taken from follicles >10mm [221].

Our research went a stage further from published data in an attempt to examine augmented LH and insulin response in PCOS thecal cultures. Examination into this effect has been shown by Nahum *et al.*, (1995) in non-PCOS theca *in vitro* using a

similar serum free monolayer system to establish the cultures [222]. They found that with the addition of 10ng/ml LH to similar insulin treatments, androstenedione was increased 2-3 fold by cultured theca. Similarly to our findings in non-PCOS cultures the augmented effect of LH and insulin was significantly increased over basal and LH stimulated androstenedione secretion. Non-PCOS secretion was seen to be increased 4-fold with the addition of insulin and LH (10 and 10 ng/ml) and correlated with increases in insulin with 100ng/ml insulin leading to a 8-fold increase in androstenedione secretion. These values were seen to be substantially increased in PCOS cultures, rising from basal levels of 6pmol/1000 cells to 80-100pmol/1000 cells (10/100ng/ml insulin). These effects not only support the augmentation of insulin +LH but also the increased sensitivity shown within PCOS theca. These levels also show a dramatic contribution to physiological androgen and emphasise the involvement in PCOS ovarian androgen synthesis in the condition. It should be noted however that variations may be present *in vitro* to those seen *in vivo*.

In summary, our results show that following examination of non-PCOS and PCOS theca primary cultures, variations do exist. Whether this is within the direct physiology, specifically protein expression; or in the metabolic response of the cell to hormonal influences, the differences found have direct implications to PCOS. Furthermore the effects on the associated symptoms can be seen in the exaggeration of androgen secretion.

8.0 DISCUSSION

Four main theories of pathophysiology have been proposed to explain the mechanisms that cause PCOS. These are the ovarian, adrenal, metabolic and neuroendocrine hypotheses. Both the ovarian and adrenal hypotheses focus on dysfunction within these two organs resulting in increased androgen secretion [147, 373-375]. The neuroendocrine hypothesis involves hyper secretion of basal LH, classically indicative of PCOS, and acting as an associated agonist for increased androgen synthesis [376]. Finally, the human metabolic hypothesis indicates an association between insulin resistance and hyperandrogenism in women, mainly related to obesity and subsequent decreases in insulin sensitivity [185, 234, 377]. Most of the research into these areas has identified aspects of dysfunction that contribute to our understanding of PCOS but no true primary cause for this syndrome has been found.

The research presented in this thesis has attempted to incorporate aspects of these four theories with a view to bridge the hypotheses and direct future work. Whilst doing so it has remained focused on the importance of androgen excess as a key endocrine abnormality.

Androgen excess is considered the key biochemical abnormality associated with PCOS, usually manifesting during puberty [120, 353]. Research by Corbould *et al.*, (2008) suggests hyperandrogenemia encourages the development of PCOS as well as metabolic disturbances associated with PCOS. Corbould also hypothesizes that reduction of androgen levels may improve insulin sensitivity and showed that decreasing androgen levels or blockade of androgen action improved insulin sensitivity when examined through euglycemic hyperinsulinemic clamps [378]. Furthermore, administration of testosterone into female rats induced skeletal muscle insulin resistance supporting the associated influence androgen may have on decreasing insulin sensitivity [378]. This supports the belief that insulin resistance is a secondary presentation associated with PCOS and although common, it is not found in all patients, with a study into a large PCOS population by DeUgarte *et al.*, (2005) estimating around 64% prevalence [379]. Similarly those showing insulin resistance

tend to acquire compensatory hyperinsulinemia. With this understanding it is believed that insulin resistance is not essential in the development of PCOS and that androgen dysregulation is intrinsically fundamental to the pathogenesis of PCOS [380-382].

The importance of androgen synthesis is seen throughout research into PCOS and ovarian biosynthesis and has been shown to account for around 50% of production in non-PCOS women, with these levels found to be significantly increased in PCOS women. Although the mechanism for this is not established, a variety of enzymes and signaling pathways have been examined. 17α-hydroxylase/17,20 lyase has been theorized to be involved as it plays a key role in androgen synthesis. This enzyme has been shown to respond positively to a co-stimulatory effect of insulin and LH (typically elevated in PCOS) within ovarian androgen biosynthesis [45, 91].

Although adrenal and ovarian sources account for the majority of secreted androgen (specifically androstenedione), it has been shown that peripheral tissues such as epithelia and adipose [208, 284, 383-385] also contribute to steroid conversion. With obesity considered a condition associated with sex hormone imbalances [386] and with its occurrence in between 38-88% of PCOS patients [387, 388], its association as a major contributory factor particularly to androgen excess and therefore warranting further investigation in our study. Although it is understood that androgen plays an important role in body fat distribution and also that androgen excess is linked to central obesity [19], it is uncertain whether obesity is implicated in the pathogenesis of PCOS. However, it is understood that obesity does aggravate androgen excess in PCOS. Evidence also exists showing that levels of SHBG are found to be lower in women suffering from PCOS. With an inverse relationship seen between plasma SHBG and abdominal obesity this maybe further indication of a relationship between obesity and PCOS [190, 389]. Furthermore research has shown that exogenous treatment with androgens in female to male transsexuals has shown evidence that androgens can lead to increased abdominal fat accumulation [390].

Thus evidence is consistent with obesity being a risk factor in the development of PCOS pointing to a key role of adipose in PCOS. With the relatively recent discovery of adiposes ability to act as an endocrine organ through the discovery of leptin in 1994 [225], examination into mechanisms of regulation and metabolic function are of clinical importance in metabolic disorders of endocrine dysfunction. This thesis

therefore has examined both metabolic function and regulation and suggests the importance adipocytes may have in both PCOS and non-PCOS. This has allowed for both mapping of non-PCOS adipose function; and understanding of the mechanisms that may be involved in PCOS. We further supported these examinations through a comparative study of ovarian thecal steroidogenesis and their involvement in PCOS dysfunction under the same hormonal conditions typically found in PCOS patients. This was all achieved through *in vitro* examination of the two tissue types (ovarian/adipose) using primary cultures.

The work described in this thesis attempted to examine:

- Variations / dysfunction between non-PCOS and PCOS adipocytes
- An association between PCOS adipocytes and hyperandrogenemia

The central findings of this work are:

- Adipocytes derived from non-PCOS and PCOS women and maintained *in vitro* differ on the basis of their morphology, rates of differentiation and proliferative abilities (Chapter 4, 5). PCOS adipocytes were shown to have increased size, increased rates of proliferation and decreased rates of differentiation compared with those from non-PCOS women.
- Demonstration of altered metabolic responses in adipocytes under conditions designed to mimic PCOS *in vitro*. These responses included proliferation, protein expression and hormone secretion. Both non-PCOS and PCOS adipocytes were seen to have reduced rates of LHR expression on LH treatment. Non-PCOS adipocytes showed reduced rates of proliferation not seen in PCOS adipocytes under LH treatment. Non-PCOS and PCOS adipocytes were seen to have increased LHR expression following incubation with insulin. Proliferation of PCOS adipocytes was increased with insulin treatment compared with adipocytes from non-PCOS women. Interestingly, expression of CYP17 in PCOS samples was increased following incubation at lower levels of insulin than that seen in non-PCOS adipocytes.
- Secretion of progesterone and androstenedione from non-PCOS and PCOS adipocytes *in vitro* and their endocrine modulation. Non-PCOS adipocytes

were seen to have increased androstenedione secretion on insulin treatment. Whereas PCOS adipocytes remained unaffected by insulin stimulation and secreted constant levels of androstenedione similar to that seen by insulin stimulated non-PCOS adipocytes.

- The involvement of the cAMP pathway in mediating adipocyte hormone secretion in response to insulin and LH levels associated with PCOS, which requires further exploration (*see 8.2*)

8.1 Comparing non-PCOS and PCOS adipocytes

Investigation of adipocytes *in vitro* faces immediate limitations. As with virtually all culture-based studies, *in vivo* conditions can never be truly replicated. Following any length of culture, further adaptions by the cell type to its *in vitro* environment can occur making any results less representative of physiological conditions or response [391-393]

With the limited ability of adipocytes to proliferate following maturation [223], and due to the difficulty of culturing mature adipocytes directly from biopsy, it was necessary to culture preadipocytes then to differentiate them into viable cultures. The method of differentiation using a cocktail of dexamethasone, IBMX and insulin (*see 3.2.2)* and as reported by others ([247, 253]), was found to produce lipid droplet formation in the adipocytes indicative of a mature phenotype. This enabled characterization of PCOS and non-PCOS mature adipocytes and examination of morphology and behavior. The morphological changes of the cultures showed qualitatively, increased size due to lipid droplet formation within PCOS adipocytes. Furthermore examination of differentiation showed that PCOS preadipocytes matured at a slower rate than non-PCOS over a period of 4-6 weeks. Our investigation focused on subcutaneous abdominal biopsies as previous studies have shown a relationship exists between PCOS and increased abdominal fat accumulation [380]. Secondary to this, reduced serum adiponectin and lower LPL activity has been seen in PCOS abdominal adipocyte biopsies and this may be indicative of metabolic disturbances specific to PCOS [130, 232, 257]. These studies support the possibility of dysfunction in PCOS abdominal preadipocytes as was seen in our results through slower differentiation. Our results have shown slower differentiation and suggested increased

lipid droplet size and although the size variation is qualitative data it is not the first documented occurrence of this finding [232, 257]. I propose one possibility for these findings as a reduction in the ability of PCOS adipocytes to undergo lipolytic expansion as a result of accommodating excess energy intake by the individual and leading to alteration in the adipocytes metabolic processes. This could eventually result in serious health issues including type II diabetes, over production of hepatic glucose and reduced insulin secretion [259, 264]. This defect would also be expected to result in increased insulin resistance. As all these symptoms are seen within PCOS patients and with the knowledge that PCOS patients tend to have an increased energy intake due to insulin resistance, this observation in PCOS adipocytes may therefore be substantiated and seen to aggravate the condition dramatically. With this in mind we can begin to understand how studies showing weight loss can reduce PCOS symptoms removing the pressure of lipid accumulation within muscle and pancreatic stores and allowing resumption of insulin action. In our model the defect would be seen in our primary PCOS cultures due to the low passage of the cultures examined and seen in the reduced speed of differentiation. This theory could be further examined by further passage of the cultures in low serum conditions representative of reduced energy intake and may lead to resumption of normal metabolic function when examining differentiation.

Morphological variation seen following differentiation, were particularly interesting. The increased adipocyte size seen in *in vitro* PCOS cultures shown by Manneras-Holm is indicative of increased storage through availability and although seemingly contradictory to the slow development from preadipocytes this may support the theory of excess availability forcing preadipocyte development to slow down to accommodate for existing hypertophic mature adipocytes as described by Faulds *et al.*, (2003). It should be noted that we saw similar results within non-obese PCOS patients, which has also been suggested by both Faulds *et al.*, and Moro *et al.*, through examination of catecholamine-induced lipolysis in subcutaneous samples. Faulds results showed reductions in triglyceride mobilization and may therefore offer an explanation as to the increased size of lipid retention following maturation [235, 265]. This may also go someway in explaining later development of obesity within PCOS patients. Faulds examinations hypothesized decreased $\beta 2$-adrenergic receptor as playing an important role in this dysfunction. This catalytic component of protein

kinase A (PKA) would therefore decrease lipolytic activity through inactivation of the protein. Our results also showed variation in proliferative responses between non-PCOS and PCOS cultures with hyperplastic behavior shown by PCOS cells. A combination of these traits in *in vivo* abdominal PCOS adipocytes could contribute to the increased abdominal obesity typically seen within PCOS patients.

With the predominance of increased LH levels within PCOS we examined the possibility of an association with adipocyte metabolism including proliferation and the insulin pathway. Examination of all samples taken from non-PCOS and PCOS adipose biopsies allowed us to confirm the expression of the LH receptor at all stages of differentiation with no variation in expression in PCOS cells. As a relatively novel area of investigation, the relationship between LH and adipocyte metabolism is yet to be fully understood but implicates LH in influencing adipocyte behavior. Furthermore our results have suggested that LH significantly inhibits proliferation of non-PCOS adipocytes determined using mitochondrial activity *in vitro* as a marker of cell viability. We also found PCOS adipocytes failed to elicit the same response, with LH having no effect on proliferation. This suggests that a dysfunction may exist in PCOS adipocytes that removes possible LH regulation over proliferation. A plausible mechanism could be the activation of cAMP via PKA with cAMP-dependent activation having been shown to inhibit proliferation in cells including adrenocortical and smooth muscle, through possible increased sensitivity to TNFα-induced apoptosis [394-396]. It should be noted that the proliferation experiments focused on pre adipocytes and therefore did not include media supplementation with IBMX which, based on our theory, may have affected the actions of LH through increases in intracellular cAMP.

The β2-adrenergic receptor dysfunction hypothesized to exist within PCOS adipocytes may therefore be associated with both differentiation and proliferative responses seen in the PCOS phenotype and is a target area for further examination.

8.2 Androgen-adipocyte relationship in both non-PCOS and PCOS cells

PCOS is characterized predominantly by hyperandrogenism, however 50% of PCOS women show phenotypic abdominal obesity with many features of the metabolic syndrome such as insulin resistance, hyperinsulinemia, diabetes and cardiac disorders

occurring inconsistently [397]. With the aetiology of PCOS still unknown, it has been suggested that obesity may play an important role in its development [157, 398]. With obesity believed to be responsible for insulin resistance and hyperinsulinemia in PCOS through increased amounts of non-esterified fatty acids, glycerols, hormones and pro-inflammatory cytokines [399], there maybe a subsequent link to its key involvement in symptomatic hyperandrogenemia. Additionally studies in adipose/androgen associations have suggested that increased activity within the opioid system and hypothalamic pituitary adrenal axis [400] as well as decreased SHBG synthesis and high dietary lipid intake [401], may encourage increased androgen synthesis.

Our findings have shown evidence of secretory androgen levels *in vitro* not only through its presence within the culture media but also the presence of the precursor progesterone and CYP17; the key enzyme involved in androgen synthesis. This may suggest not only the existence of androgen processing but also a biosynthetic pathway within adipocytes for androgen. If this were the case it would give avenues for examination into additional influx of androgens possibly associated with PCOS and may also point to an area that may be involved in hyper secretion. Although the levels determined were comparatively lower than those seen in gonadal and adrenal androgen secretion by *in vitro* cultures as shown within our results in Chapter 7 as well as findings by Gilling-Smith *et al.*, (1994) and Hughes (1988), they were seen to be significant against negative controls. When considered in a physiological model, levels of secretion would correlate to increases in adipose tissue resulting in a cumulative response typically associated to PCOS weight gain [221, 267]. Furthermore the levels found from all these cell types correlate to physiological levels via their physiological contributions whereby ovarian and adrenal levels have been shown to supply the greatest inputs of physiological androgen [342]. Whether these secretory levels are seen to be for auto/intracrine function is yet to be established, however the results suggest a localized androgen synthesis and further the understanding of the endocrine function of adipose. Our comparison of secretory androstenedione levels from non-PCOS and PCOS adipocytes showed that no variation within levels reached exists under our *in vitro* conditions. This may suggest that no dysfunction exists within PCOS adipocytes androgen synthesis however further investigation into not only determining the pathways involved but also

conditions representative of the *in vivo* environment would be necessary to support this.

The physiological levels of insulin and LH found in PCOS patients are typically elevated. Cells often respond to insulin through increases in metabolic response such as proliferation or increased rates of biosynthetic pathway activity; which has been seen within androgen biosynthesis in both thecal and adrenal cells [398, 402]. This has been further examined under hyper physiological insulin treatment ranges *in vitro* representative of PCOS [403]. Munir *et al.,* (2004) showed significant increases in thecal steroidogenic enzymes key to androgen synthesis [45, 357]. With our findings suggesting an additional source of physiological androgens via adipocyte cell synthesis and, secretion, the examination of insulin on adipocyte metabolism allowed for a greater understanding of adipose function. It also enabled us to see how any dysfunction that may exist in PCOS adipocytes may be affected by these biochemical variations. Insulin treatments resulted in an increase in androstenedione secretion in non-PCOS adipocytes at 10ng/ml, which remained the same regardless of further increases in insulin. This result was similar to that seen in insulin's action on gonadal and adrenal tissue androgen synthesis suggesting a similar mechanism of regulation may exist in adipocytes. From this similar hypothesis to those found in ovarian and adrenal steroidogeneisis, may therefore be drawn to the involvement of hyperinsulinemia and its associations to increased androgen secretion that results in hyperandrogenism. With this in mind the link between insulin resistance and obesity would therefore give a possible foundation to increased androgen secretion. Although our examination of PCOS adipocyte androgen secretion showed no variation within the levels of androstenedione secretion against the non-PCOS culture we found that they did not react to insulin stimulation. Our results therefore suggested that the PCOS adipocytes were in a constant state of stimulation and dysfunction may exist in androstenedione regulation as a result of PCOS hyper stimulation.

To further support the possibility of an androgen synthetic pathway in our adipocyte cultures, our results showed that levels of progesterone decreased (1pmol/1000 cells) at the same insulin treatment level that caused an increase in androgen secretion. This finding may indicate progesterone's involvement in the androgen biosynthetic pathway. The increase in androgen synthesis as a result of insulin stimulation would

therefore require a greater supply of precursors. In addition to this we found the presence of the enzyme CYP17, necessary for the conversion of progesterone to androstenedione. This enzyme did not vary in expression between non-PCOS and PCOS samples suggesting that it is unlikely that a dysfunction exists at this stage of a steroidogenic pathway. Furthermore this reinforces our evidence that no variation in adipocyte androstenedione production exists between non-PCOS and PCOS in the absence of treatment. However lack of variation may suggest CYP17 is expressed at similar levels to those found in non-PCOS but activity is reduced.

Examination of non-PCOS and PCOS adipocyte samples showed no differences in increased insulin stimulation of androstenedione secretion. Overall variation in insulin treatment did not cause any variation in levels of PCOS and non-PCOS androstenedione secretion and a constant level was maintained within our treatment range (2.8pmol/1000 cells). This was supported by stable progesterone levels, as seen in non-PCOS samples. If representative of *in vivo* activity this would indicate a constant state of androstenedione secretion regardless of increased stimulation, which would enhance serum androgen levels. Furthermore it would also support the insulin resistant dysfunction already associated with obesity and PCOS, particularly in adipocytes. Our ELISA results did show possible room for error and to fully support this theory further repetition of these results would be required as our sample size was limited. We did however attempt to support this through our protein expression findings.

Documented evidence has shown that LH increases ovarian and adrenal androgen synthesis [32, 91]. With hyper-secretion of LH often found in PCOS patients its involvement in the mechanisms surrounding symptomatic hyperandrogenism has been of particular interest. This factor, coupled with adipocyte androgen secretion, may further increase physiological androgen levels in PCOS patients and it has been determined that LH increases non-PCOS ovarian androgen secretion significantly. In addition to this, LH and insulin treatment has been shown to increase non-PCOS ovarian androgen secretion 8-fold [91]. Examination of this influence on adipocyte androgen secretion showed that androstenedione increased with insulin treatment, although no variation was seen with LH treatment alone. However our findings showed this response was reduced in the PCOS samples. This may suggest that the

dysfunction seen in proliferation and differentiation also exists in androstenedione secretion, however our examinations gives no evidence of an association and would require further work to confirm this. With the influence of insulin seen to increase androstenedione in non-PCOS adipocytes it may suggest that the loss of effect dysfunction arises as a result of the onset of PCOS. This may be true as abnormal fat deposits suggestive of changes in adipocyte activity are associated with PCOS as are the effects of increased androgen exposure that lead to changes in adipocyte metabolism through reduced proliferation and differentiation [298].

With such a variation often seen in the presentation of symptoms found in PCOS our determination of a more active and key role of adipocytes within PCOS dysfunction may begin to address a more central pathogenesis of the condition. From this, we may therefore gain a better understanding of the correlation often seen in weight fluctuations of PCOS patients and the severity of the conditions or symptom.

8.3 Ovarian theca cells in non-PCOS and PCOS patients

Hyperinsulinemia and PCOS are often associated [302, 398]. Increased insulin action is believed to lead to increased synthesis of ovarian androgen, with both increased insulin and increased androgen considered diagnostic features of the syndrome. Research has shown an increase in the number of steroidogenic theca cells of PCOS patients when compared with controls [129], moreover it has been shown that PCOS theca interstitial cells are in a state of hyper stimulation in those samples taken from patients with biochemical abnormalities including hyperinsulinemia and hyper secretion of LH [221]. Additionally, examination into the steroidogenic pathways involved in androgen biosynthesis has shown increased expression of key enzymes including StAR, CYP11A and 3β-HSD in theca cells within PCOS [404] All these factors are suggestive of a key involvement of PCOS ovarian theca in the hyperandrogenic component central to the condition. Androgen is also increased at basal/untreated levels of secretion in PCOS cultures compared to controls, further emphasizing a dysfunction that would lead to a greater influx of circulatory androgen. With ovarian androgen synthesis responsible for 30-50% of overall androgen secretion, further increases in PCOS theca and/or activity can be seen as a major physiological addition that would upset androgen homeostasis. The second

component of this thesis therefore looked to examine variations in PCOS ovarian theca *in vitro* and the physiological factors that may affect androgen steroidogenesis including insulin and LH. It also looked briefly into the pathways that may be involved in androgen hyper secretion in order to determine whether dysfunction exists and at what point of synthesis.

The key findings of this thesis derived from work described in Chapter 7 on theca and therefore include:

- CYP17 expression is increased in PCOS theca under basal conditions and that augmentation of insulin and LH leads to increases in *in vitro* theca proliferation and expression of CYP17 in both non-PCOS and PCOS samples.
- Demonstration of lack of variation between basal and insulin stimulated *in vitro* thecal androstenedione secretion.
- Demonstration of increased *in vitro* thecal androstenedione secretion under LH treatment in both non-PCOS and PCOS theca cells which was further increased through augmentation of insulin+LH.
- Increased androstenedione secretion by *in vitro* PCOS theca cells under augmented insulin+LH treatment over non-PCOS theca cells under the same conditions.
- Findings suggestive that insulin is acting through the PI3-K pathway in thecal androstenedione synthesis. This however requires further evidence and examination.

With the importance of ovarian steroidogenesis as a source of androgen production in premenopausal women, research has focused on thecal cell dysfunction in PCOS as a possible root for androgen synthesis. In the past, much of this research has been carried out on direct tissue incubations [398] or freshly pooled theca cells capable of steroidogenic activity. These sources have limited the area of examination through their inability to be sustained or limited cell yields [43, 221]. However, the production of a sustainable *in vitro* monolayer culture system by McAllister *et al.*, (1989) allowed better insight into the steroidogenic function of theca cells as well as variations that may occur within PCOS samples [60]. Using the protocol we were able to adapt and reproduce the techniques described and maintain cultures *in vitro* (*see*

3.2.1). Difficulties did arise in establishing PCOS theca cultures outside of the technique in maintaining cell numbers in the initial passage. This was remedied by increasing insulin and the addition of a high serum supplement and was used throughout all our cultures. This high-energy requirement may reflect the initial *in vivo* environment. As discussed in *8.1* PCOS patients typically have high physiological insulin levels (hyper insulinemia) as a result of insulin resistance and also increased energy intake. This theory seems concurrent with reported findings and I was unable to find any protocols that used serum free to establish a primary PCOS theca culture, and those that did used high insulin, glucose supplements within the media. Although many methods existed for non-PCOS primary theca cultures that were serum free as stated in the original protocol created by Magoffin and Erickson (1982). Following establishment of the primary cultures, we used immunofluorescence (*see 3.5.1*) as a marker for contamination by other cell types and oil red O staining of lipids. Through this, we were able to examine morphological variations including size and lipid formation (size and number) in theca as well as adipocytes (*described 8.1*) that may exist across non-PCOS and PCOS sample groups. Published results examining rat thecal cultures induced with estradiol valerate (EV) to mimic PCOS [154], reported larger cell morphology and number within the PCOS group. Our results also showed increased cell size within our PCOS cultures and therefore suggested this hypothesis may be valid within our human cells. Variations in theca size maybe as a result of larger lipid droplet formation and number. These characteristics may be associated with the increase in steroidogenic activity documented by Manna *et al.*, (2003) with lipid droplets used as cellular storage for precursors such as carbohydrates, progesterone and cholesteryl esters [358]. Increased size may therefore dictate increased demand or possibly decreased demand without decreases in production. Alternately this may point to similar dysfunction as suggested within our adipocyte work whereby studies into non-obese PCOS women showed decreases within catecholamine-induced lipolysis in subcutaneous abdominal adipocytes suggestive of resistance [235, 265]. This would therefore prevent mobilization of triglycerides increasing lipid droplet storage, dictating the subsequent size of the cell. Studies into therapeutic agents such as metformin have shown that through increased insulin sensitivity, symptoms associated to the hyperandrogenic conditions of PCOS are reduced and that metformin can directly inhibit androgen

production in human theca [405]. Nestler *et al.,* (1998) also showed that administration of metformin allowed spontaneous ovulation [406]. Furthermore La Marca *et al.,* (2000) noted that following metformin treatment stimulated CYP17 activity was reduced [367]. This gives a great deal of information toward thecal androgen steroidogenesis and points to the importance of CYP17 activity through insulin pathway activation. Our study however found that although variations in CYP17 exist between non-PCOS and PCOS groups, variations in insulin treatment did not lead to any changes in CYP17 expression. With this in mind the effects of therapeutic insulin sensitizers such as troglitazone and metformin would therefore not be effective through their effects on insulin behavior alone. The agonistic effect of LH on ovarian androgen synthesis has been documented in both non-PCOS and PCOS patients. It has also been seen that the effect is more sensitive in PCOS theca cells [91]. More importantly the augmented effect of insulin and LH in both non-PCOS and PCOS samples is considerably greater than basal levels [221]. Our results can confirm this effect within PCOS theca and showed significant effects through augmentation of LH and insulin when compared to individual treatments and on comparison to non-PCOS cultures. From this we can hypothesis that the reduction in hyperandrogenic symptoms often seen from the use of insulin sensitizing agents maybe linked to a process that helps to reduce this augmentation. Furthermore treatment of PCOS with metformin has shown to re-establish feedback control of testosterone through LH secretory rates and therefore improving neuroendocrine abnormalities such as LH hyper secretion often associated to PCOS [407].

It has been proposed that in PCOS patients presenting with hyperinsulinemia and hyperandrogenemia the augmentation of LH and insulin is central to excess androgen stimulation [45, 221]. However we have shown that the effects of LH work independently in stimulating increases in androstenedione secretion above basal levels (*see 7.4.4*). Furthermore, our examination of both normal and supra-physiological treatments shows that normal circulatory insulin levels can cause significant increases in androgen stimulation. This may go someway to explain why PCOS can present without hyperinsulinemia and that thecal dysfunction central to the pathogenesis can lead to symptomatic increases in insulin. This coupled with our work with adipocyte cultures may suggest that gradual increase in physiological androgen can cause central obesity and adipocyte dysfunction that results in aggravated increases in

insulin further encouraging both adipocyte and thecal androgen secretion through LH insulin augmentation.

With the importance this dysfunction therefore poses with the condition, pinpointing the exact mechanism is therefore necessary. Munir *et al.,* 2004 has confirmed thecal insulin action on steroidogenic androgen synthesis through the insulin receptor and studies have shown that no abnormality exists in either function or expression in PCOS theca. Munir also examined the involvement of two key pathways in normal theca cultures and determined that PI3-K rather than MAPK was primarily involved in CYP17 activity [45].

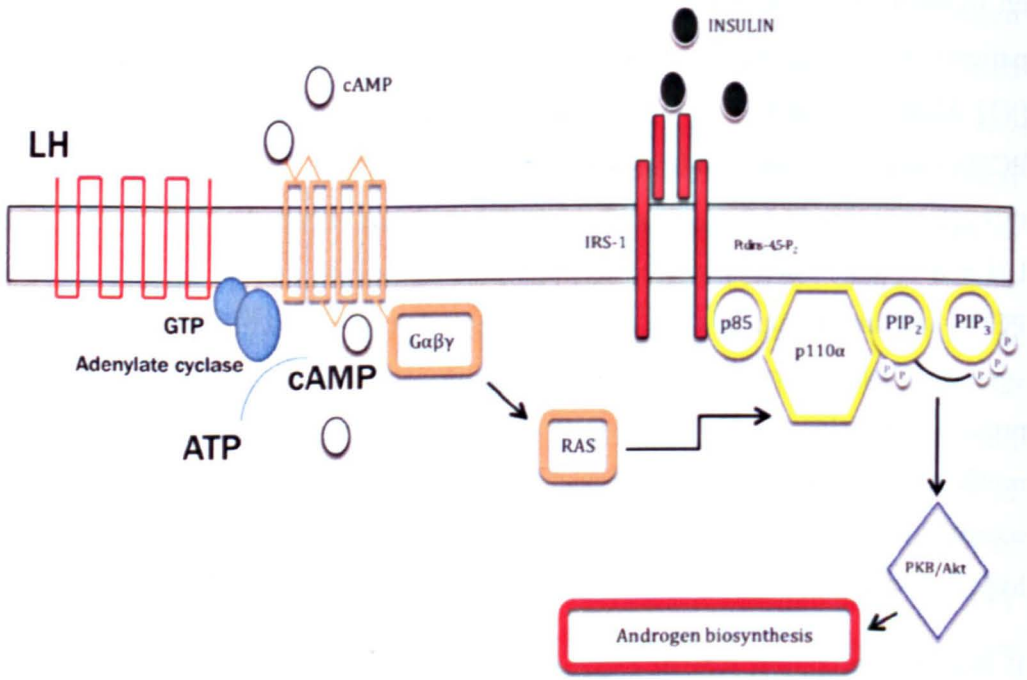

Figure 131. Suggested pathway showing cAMP and insulin co-activation of PI3-K leading to subsequent androgen biosynthesis.

The stimulatory effects of insulin on CYP17 activity was also seen to be dependent on the co-activation of the cAMP signaling pathway [45] which again supports the importance of the involvement of LH in increasing steroidogenic activity. **Figure 131** shows the pathway that links LH and subsequent cAMP activation to PI3-K and may therefore be associated with the involvement of insulin and consequent co-activation of steroidogensis. Luteininsing hormone / chorionic gonadotropin activates multiple signal transduction systems [311, 408]. Most of the sequential steps involved after

hormone binding, until G protein activation on the inner face of the plasma membrane remains poorly understood. However in many experimental systems, LH or hCG binding to LHR results in activation of both protein kinase A and protein kinase C [311], and at relatively low concentrations of LH and hCG protein Gs' appear to be the preferred signaling pathway that results in a rapid increase in the intracellular concentration of cAMP.

If the pathway in **Figure 131** did operate, the inhibition of PI3-K would lead to a reduction in both LH and insulin stimulated CYP17 and resultant reduction in androstenedione synthesis. Our results show this to be true for insulin stimulation alone in both non-PCOS and PCOS through reduced CYP17 expression. This study therefore requires further examination into the effects PI3-K inhibition may have on LH stimulation and combined LH and insulin on CYP17. Moreover this would require support through measurements of androstenedione secretion under these conditions. From this we could determine the importance CYP17 may be as a target for dysfunction within this pathway and subsequent disturbances within androstenedione synthesis and also include the involvement of LH within this pathway.

This thesis has identified the potential importance of adipose tissue as a source of androgens as well as furthering the current understanding of ovarian androgen synthesis within PCOS. Although there are few reports, this thesis describes for the first time the presence and modulation of androgen synthesis in both tissue types under the influence of insulin and LH within PCOS. In doing so we have highlighted a potentially new pathway in adipocyte metabolism and therefore increased the understanding of PCOS with a hope of assisting in its management.

9.0 FUTURE WORK

9.1 LH and the MAPK pathway

Through the examination of the findings in this study and based on previous work I have begun to examine the possible pathway in thecal steroidogenesis that may link LH activation of androstenedione and harbor an area of dysfunction that requires focus for future work. Ras proteins are located on the inner surface of the thecal plasma membrane and are GTPases involved in receptor-mediated signal transduction pathways (**Figure 132**) transmitting extracellular signals that promote growth, proliferation, and differentiation. The signaling cascade starts from the plasma membrane where the agonist binds to its enzyme-linked receptor. Receptor-linked tyrosine kinases such as the epidermal growth factor receptor (EGFR) are activated by extracellular ligands and binding of EGF's. This leads to phosphorylation of the intracellular domain of the receptors, activating guanine exchange factors (GEFs)[409], such as son of sevenless (SOS) [410]. GEFs allow the exchange of GDP to GTP activating Ras. The major downstream target of Ras-GTP is mitogen-activated protein kinase (MAPK), but it also activates phosphatidylinositol 3-kinase (PI3-kinase)(Chapter 8 Figure 1), and Ras-related guanine nucleotide dissociation stimulator (RalGDS). Activation of MAPK occurs through specific phosphorylation of Raf, which is activated on the plasma membrane by Ras-GTP. Raf phosphorylates mitogen-activated kinase 1/2 (MEK1/2 kinase), in turn activating the extracellular regulated kinase 1/2 (ERK1/2 kinase or p44/42 MAPK) by phosphorylation.

The second class of enzymes in the ERK cascade, the MEKs, phosphorylate threonine and tyrosine residues. MEK1 and MEK2 are involved in ERK signaling and activated via their only substrate, Raf kinase. Inactivation of the ERKs is through de-phosphorylation via serine threonine or tyrosine phosphatases [411, 412]. ERK1/2 kinase phosphorylates a variety of downstream targets, which results in changes in gene expression and the catalytic activities of enzymes [413].

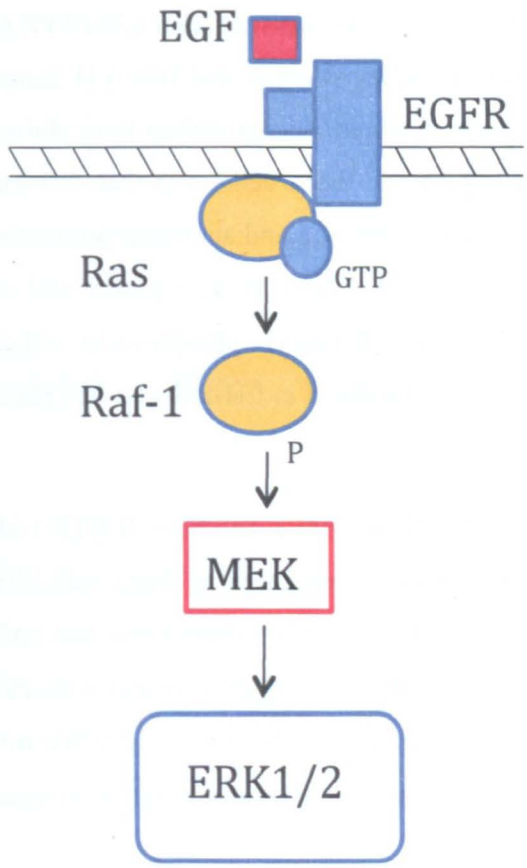

Figure 132. RAS/RAF/MEK/ERK signalling cascade. ERK activation through MAPK initiated by many extracellular cues such as extracellular growth signals (shown here via EGF receptor) and tyrosine kinases. The signal is transduced from RAF to ERK through GTP bound RAS activation.

A recent study showed that both MAPK kinase (MEK1/2) phosphorylation and extracellular signal related kinase (ERK1/2) phosphorylation were reduced by 50% in PCOS cells compared with that in normal cells [414]. A reduction in phosphorylation within this pathway may therefore lead to an activation or reliance on an alternate pathway and with the involvement of LH and insulin acting through the convergence of two pathways this maybe the case. This is further supported with the reduction in MEK1/2 and ERK1/2 phosphorylation showing association with increased thecal CYP17 gene expression and androgen biosynthesis [45]. Treatment of theca cells with the MEK inhibitor PD98059 has been also shown to augment thecal CYP17 gene expression and androgen biosynthesis [414]. This may suggest that if this pathway is involved in androgen synthesis it may act to regulate as well as stimulate steroidogeneisis. The extent to which the other components of the MAPK pathway contribute to overall androgen biosynthesis requires further examination.

Although LH has been reported to activate the cAMP/PKA pathway and the ERK/MAPK pathway in theca cells, whether and how LH stimulates the PI3-K/Akt cascade in theca cells remains unclear. Recent studies have shown LH stimulates Akt phosphorylation in cultured bovine theca cells, and that activation of PI3-K/Akt is involved in CYP17A1 mRNA expression and androgen production in theca cells thus supporting LH activation of PI3-K [415]. H89, a potent and selective inhibitor of PKA, did not affect LH-mediated changes in phospho-Akt, indicating that a pathway distinct from that of the PKA is involved in LH-induced Akt phosphorylation in theca cells.

In contrast to the PKA inhibitor, the MEK inhibitor (U0126) blocked LH-mediated Akt phosphorylation and androgen production in theca cells [59]. We are therefore aware that LH stimulates CYP17A1 mRNA expression and androgen production in theca cells via activation of the PI3-K/Akt pathway and with the involvement of the MAPK pathway. Further supporting a combined pathway that may not only stimulate but regulate and may work independently and through individual stimulating factors as seen with LH.

While the precise mechanism for the activation of PI3-K pathway by LH in theca cells is not known, it is possible that the LH-induced phospho-Akt up-regulation may involve MAPK-mediated down-regulation of phosphatase and tensin homologue (PTEN; a tumor suppressor which negatively regulates Akt phosphorylation). In this context, it has been shown that PI3-K is required for estradiol-stimulated hepatic cell growth and that the MAPK pathway reduces the level of PTEN, allowing estradiol-induced phosphorylation of Akt [416]. However my focus would be on GTP bound RAS due to its interactions within the pathway through its activation of class Ia kinases as shown by Downward, 1998 [417, 418].

Clarification of the LH-mediated intracellular signaling events is essential, along with consideration for cross talk and compensatory mechanisms between pathways. This will allow for better understanding of not only ovarian physiology, but also of the pathophysiology of PCOS.

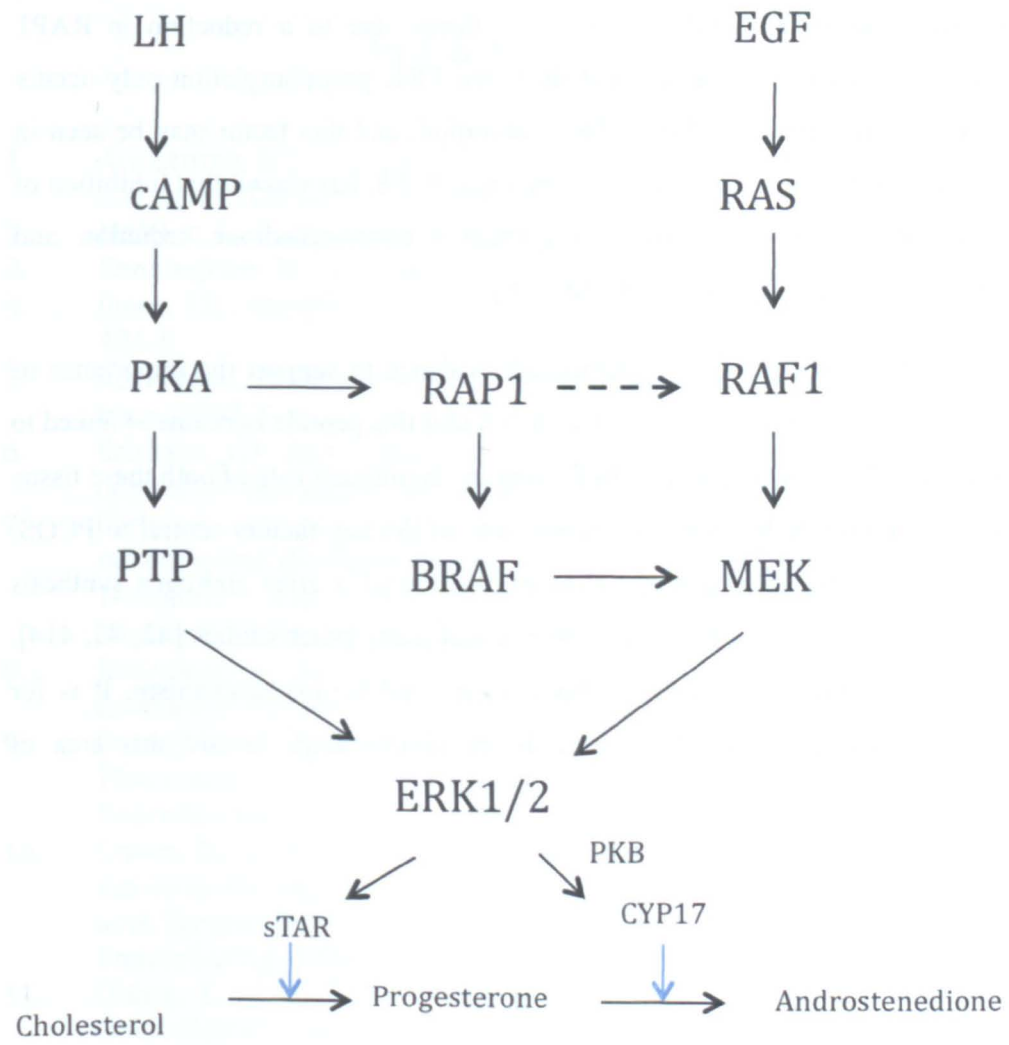

Figure 133. Suggested pathway for LH activation of theca androgen biosynthesis acting through cAMP activation of MAPK.

Studies in granulosa have shown elevated cAMP maybe associated with ERK activation [419]. PKA forms inactive RAP1 inhibiting RAF1 and preventing MEK phosphorylation of ERK and subsequent androgen synthesis [420]. I propose increased cAMP may allow phosphorylation of cAMP-responsive guanine nucleotide exchange factors (Epac1 and Epac2). Upon binding of cAMP, Epac1/2 rapidly activate Rap1, which subsequently promotes activation of B-raf and the rest of the ERK cascade (*see* **Figure 133**)[421].

cAMP has been shown to directly activate RAP1 [421] this would subsequently lead to BRAF activation and MEK phosphorylation (LH mediated androgen synthesis). Inhibition of PKA would prevent RAP1 formation. This would therefore lead to the

eventual return to RAF1 inhibition of the pathway due to a reduction in RAP1 formation. Studies have shown that LH mediated ERK phosphorylation only occurs within 24hrs of LH exposure during PKA inhibition, and this factor may be seen in effect within our theory. Furthermore Tajima *et al.*, 1998, has shown that inhibition of cAMP activated ERK has shown to decreases androstenedione secretion and expression of Star and CYP17 expression [420].

In summary this study provides experimental evidence to support the importance of LH in its involvement in PCOS. We have shown that this peptide hormone is linked to both adipose and ovarian regulation, indicating the significant role of both these tissue types in contributing to hyperandrogenemia, one of the key factors central to PCOS. More importantly, the influence of LH in stimulation of ovarian androgen synthesis seems to be predominant in both this research and many other studies [42, 45, 414]. However little is known about the pathways involved in this mechanism. It is for these reasons that I believe this thesis directs future work toward this area of examination.

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11.0 APPENDIX

I. Solution compositions:

PBS working dilution:

200ml PBS stock, 300ml 5M NaCl, 770ml H_2O

PBS stock:

Na_2HPO_4, NaH_2PO_4, pH 7.2-7.4 with NaOH

10% Ammonium persulphate (AP):

0.1g Ammonium persulphate in 1ml H_2O

4% w/v Paraformaldehyde:

4g PFA dissolved in dH2O at 60°C. pH 7.4 using 1M NaOH. Stored at -80°C for 3months.

HBSS:

5ml of HEPES & 5ml penicillin/streptomycin in 500ml HBSS

Homogenisation buffer (ovarian):

300mM, tris base pH 7.4 25mM (Invitrogen Life Technologies), monothyioglycerol 10mM, EDTA 1mM, protease inhibitor cocktail 0.5% (v/v), 2.5% (v/v) igepal

Homogenisation buffer (adipocyte):

320mM Sucrose, 10mM Tris base pH 7.8, 50mM KCl, 1mM ethylenediaminetetraacetic acid (EDTA), 0.5% Igepal, protease inhibitor cocktail (Sigma) (1:500) supplemented with fresh phosphatase inhibitor cocktail (Sigma) 1:100.

Enzyme dissociation solution:

collagenase I, 1mg/ml, hyaluronidase 0.5mg/ml and DNase I 15kunits

Lamelli buffer/sample loading buffer:

50µl β-mercaptoethanol, 950 µl sample loading buffer (Biorad)

PBS:Glycerol:

Equal v/v of PBS:Glycerol

PSS:

119 NaCl, 4.7 KCl, 25 $NaHCO_3$, 2.5 $MgSO_4$, 1.6 $CaCl_2$, 1.2 KH_2PO_4, 5.5 glucose, 0.034 EDTA pH7.4 Stored at 4°C

Resolving gel:

12%: 4.1ml dH_2O_2, 3.3ml Acrylamide, 2.5ml resolving gel buffer (Tris base pH8.8), 100µl 10% SDS, 100µl ammonium persulfate (APS), 4µl N,N,N'N'-Tetramethlyethlyenediamine (TEMED).

Running buffer:

15.1g Tris, 94g Glycine, 50ml 10% SDS in 1L H_2O.

Solubilisation buffer:

20mM Tris base pH 7.5, 10mM EDTA, 120mM NaCl, 50mM KCl, 2.5% Igepal supplemented with fresh protease inhibitor cocktail (Sigma) (1:500) and phosphatatse inhibitor cocktail (sigma) 1:100).

Stacking gel:

4%: 3.4ml dH_2O_2, 0.85ml Acrylamide, 0.625ml resolving gel buffer (Tris base pH8.8), 50µl 10% SDS, 50µl APS, 5µl TEMED.

TBS-Tween (TBST) 0.1%:

1L TBS 1ml Tween-20

Transfer buffer:

24mM Tris Base, 80mM glycine, 20% methanol

Tris buffered saline (TBS):

12.11g Tris, 146.1g NaCl, in 5L H_2O pH to 7.4 with 5M HCl.

II. Working dilutions of antibodies

Table 1. Detailing the dilution factor for each antibody included in our studies according to which technique was used.

Antibody	Host	Type	Working Dilution & Technique	Secondary antibody & Conjugate
Alpha actin (DakoCytomation)	Mouse	Monoclonal	1:50 IF 1:50 IHC	1:50 FITC (Sigma) 1:1000 FITC (Molecular Probes) Vectorstain Elite Kit (Universal) (Vector laboratories)
Beta actin (Abcam)	Rabbit	Polyclonal	1:8000 WB	1:30000 AP (Sigma) 1:2000 AP (DakoCytomation)
CYP17a1 (Santa Cruz)	Rabbit	Polyclonal	1:500 WB 1:50 IF	1:30000 AP (Sigma) 1:2000 AP (DakoCytomation) 1:50 FITC (Sigma)
CYP17 (Abgent)	Rabbit	Polyclonal	1:200 WB 1:50 IF	1:30000 AP (Sigma) 1:2000 AP (DakoCytomation) 1:50 FITC (Sigma)
CD45 (Sigma)	Mouse	Monoclonal	1:100 WB 1:20 IF	1:30000 AP (Sigma) 1:2000 AP (DakoCytomation) 1:50 FITC (Sigma)

antiOB (R&D Systrems)	Goat	Polyclonal	1:1000 WB 1:50 IF	1:30000 AP (Sigma) 1:2000 AP (DakoCytomation) 1:50 FITC (Sigma)
LHR (H-50) (Santa Cruz)	Rabbit	Polyclonal	1:200 WB 1:50 IF	1:30000 AP (Sigma) 1:2000 AP (DakoCytomation) 1:50 FITC (Sigma)

III. Suppliers

Abcam	330 Cambridge Science Park Cambridge CB4 0FL UK Web http://www.abcam.com
Abnova	Abnova GmbH c/o EMBLEM Boxbergring 107 69126 Heidelberg Germany Web: http://www.abnova.com E-mail: sales@abnova.com
BD Transduction Laboratories	BD Biosciences 21588 Network Place Chicago, IL 60673-1215 Tel: 44.1865.78.16.66 Fax: 44.1865.78.16.27 Web http://www.bdbiosciences.com/home.jsp E-mail admetox@bd.com
Biorad	Bio-Rad Laboratories Ltd. Bio-Rad House Maxted Road Hemel Hempstead Hertfordshire HP2 7DX Tel: +44 (0)208 328 2000 Fax: +44 (0) 208 328 2550 Web: http://www3.bio-rad.com/ E-mail: uk.lsg.marketing@bio-rad.com
DakoCytomation	Dako UK Ltd Cambridge House St Thomas Place, Ely Cambridgeshire CB7 4EX Tel: +44 (0)1 353 66 99 11 Fax: +44 (0)1 353 66 73 09 Web: www.dako.co.uk E-mail: info.uk@dako.com

Fisher Scientific	Fisher Scientific UK Ltd, Bishop Meadow Road, Leicestershire LE11 5RG Tel: 01509 231166 Fax: 01509 231893 Web: http://www.fisher.co.uk
Molecular probes	**Invitrogen Ltd** Inchinnan Business Park Paisley UK PA4 9RF Tel: 0141 814 6100 Fax: 0141 814 6260 Web: http://www.invitrogen.com E-mail: euroinfo@invitrogen.com
Santa Cruz	**Santa Cruz Biotechnology, Inc.** Bergheimer Str. 89-2 69115 Heidelberg Germany Tel: +49 6221 4503 0 Fax: +49 6221 4503 45 Web: http://www.scbt.com E-mail: europe@scbt.com
Sigma Aldrich	Sigma-Aldrich Company Ltd. The Old Brickyard New Road Dorset SP8 4XT UK Tel: +44 1202 712300 Fax:+44 1202 715460 Web: http://www.sigmaaldrich.com/united-kingdom.html E-mail: ukcustsv@europe.sial.com
Tocris	Tocris Bioscience Tocris House, IO Centre Moorend Farm Avenue Bristol, BS11 0QL

	United Kingdom Tel: + 44 (0)117 916-3333 Fax: + 44 (0)117 916-3344 Web: http://www.tocris.com/contact.php E-mail: customerservice@tocris.co.uk
Vector Laboratories	3, Accent Park, Bakewell Road Orton Southgate Peterborough PE2 6XS Tel: (01733) 237999 Fax: (01733) 237119 Web http://www.vectorlabs.com/uk E-mail: vector@vectorlabs.co.uk
VWR (formerly BDH)	Hunter Boulevard Magna Park Leicestershire LE17 4XN Tel: 0800 22 33 44 Web: http://uk.vwr.com E-mail: uksales@uk.vwr.com

IV. Patient information sheet & Consent forms

At which I said: "And after the great sentence - o master - will these torments grow, or else be less, or will they be just as intense?"And he to me: "Remember now your science,which says that when a thing has more perfection,so much the greater is its pain or pleasure."
(Dante, Inf. VI, 103-111)