Polycystic Ovarian Syndrome and Adipose Tissue

Contribution of Peripheral Androgen Synthesis to Hyperandrogenism in Polycystic Ovarian Syndrome

by

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STATEMENT OF AUTHENTICITY

I declare that this thesis has been composed by myself, working under the supervision of Clinical Associate Professor, Saad Amer, School of Graduate Entry Medicine and Health, Department of Obstetrics & Gynaecology, Royal Derby Hospital, University of Nottingham, and Associate Professor Raheela Khan at the Academic Division of Obstetrics & Gynaecology, Royal Derby Hospital, University of Nottingham during the period of February 2012 to February 2015. This thesis is a record of work, which has not been submitted previously for a higher degree.

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DEDICATION

This thesis is dedicated to,

My parents, who give me unending love, and taught me patience, perseverance and dedication.

My sisters, Ahlam, Amal, Amera, and Amer, and to my best brothers, Mohamed and Ali for their support.

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ABSTRACT

Background:

Polycystic ovarian syndrome (PCOS) is the most common endocrine, reproductive, metabolic and psychological disorder in women of childbearing age, affecting 6-10% of premenopausal women.

Hyperandrogenism is the most important biochemical feature of the syndrome, which is responsible for the clinical features of PCOS, and is frequently associated with metabolic disturbance, such as insulin resistance, dyslipidaemia, glucose intolerance and hypertension, regardless of the presence of obesity. In several studies, attention has focused on androgen as the main factor for development of metabolic disturbance, which observed in women with PCOS.

The relationship between adipose tissue and the pathophysiology of PCOS, in terms of development of hyperandrogenism and its relation to development of insulin resistance with compensatory hyperinsulinemia still not fully understood. Based on epidemiological studies, the association between circulating androgen levels and insulin resistance, as well as central obesity is a direct correlation. Although ovaries and adrenal glands are the main sources of androgens, adipose tissue is also one of the most important peripheral tissues involved in the production of androgens. Adipose tissue is not just an organ with energy storage; it also has endocrine, paracrine and autocrine functions, due to secretion of active peptides, known as adipokines, and hormones, such as androgens.

In order to understand the role of adipose tissue in the development of hyperandrogenism, we hypothesised that “Androgen metabolic pathways leading to testosterone production in subcutaneous adipose tissue are altered in women with PCOS.” The excess adipose tissue androgen synthesis plays an important role in PCOS pathogenesis.

Aims:

The main aim of this study was to analyse and compare the expression level of the two key enzymes in androgens synthesis, 17-α-hydroxylase/17.20-lyase (CYP17A1) and 17-β-hydroxysteroid dehydrogenase type 5 (AKR1C3) in adipose tissue of women with and without PCOS. These are responsible for locally synthesised sex steroid hormones, mainly androgens; CYP17A1 is responsible in the production of the precursors of androgens and AKR1C3 is responsible in the conversion of inactive androgen (androstenedione) to its active form, testosterone.

In addition, to understand the mechanism of androgen production this study employed isolated pre-adipocytes cultures and *in vitro* differentiated to mature adipocytes with close regulation of the impact of insulin and LH as the most two hormones which have effect in sex steroid synthesis. In doing so, we investigated androgen synthesis by activation and expression of the main steroidogenic enzymes CYP17A1 and AKR1C3 in adipocytes of non-PCOS and PCOS women. This enabled us to study the difference in the CYP17A1 mRNA and AKR1C3 mRNA expression levels, as well as the concentration of testosterone secretion across non-PCOS and PCOS cultures. This also indicated the probability of a pathway leading to localised synthesis of adipocytes and to investigate if there is a role for PI3-K signaling pathway in insulin regulation of testosterone synthesis in peripheral adipose tissue of women with and without PCOS.

Methods:

In order to achieve these aims, subcutaneous adipose tissue samples (SC) were surgically obtained during gynecological surgery from women with and without PCOS. All participants were of reproductive age (20-45) with a BMI of 20-35kg/m^2**.** Total RNA was isolated from frozen adipose tissue samples of non-PCOS (n=8) and PCOS (n=8) after matching, using Trizol reagent method, followed by reverse transcription. Quantitative RT-PCR was performed to determine the expression of a panel of reference genes (GAPDH, ACTB, and

LPR10), and target genes (CYP17A1 and AKR1C3). Data were analysed with GenEx and compared using ΔΔCt method.

AKR1C3 protein expression in non-PCOS (n=3) and PCOS (n=3) was measured and compared by using western blot (WB) technique.

Pre-adipocytes were isolated from fresh adipose tissue samples by enzyme digestion (collagenase) method and *in vitro* differentiated to mature adipocytes, which were cultured in FCS-free medium, Recombinant insulin +/- LH+/-PI3-k inhibitor (LY294002) was added to the cell culture at different concentrations, in preparation for investigating any change in the expression of steroidogenic enzymes (CYP17A1, AKR1C3) by RT-PCR, after extraction of total RNA

The supernatant was collected for testosterone measurement before and after treatment using enzyme-linked immunosorbent assay (ELISA).

Results:

Of the reference genes testes, GABDH, ACTB, and LRP10 were shown to be consistently expressed across the PCOS and non-PCOS women. The mean± SEM relative expression level of AKR1C3 mRNA in PCOS adipose tissue was 15.1± 2.0, which was significantly (P=0.0003) greater than that (3.3±1.1) of non-PCOS women. However, the expression level of CYP17A1 mRNA was not significantly (p=0.56) different between the two groups. AKR1C3 protein expression level was less expressed in PCOS and there was no significant (P>0.05) difference in the protein expression between two groups.

CYP17A1, AKR1C3 and testosterone were significantly higher in PCOS (n=5) versus the non-PCOS (n=5) in treated and un-treated cultures. Insulin did not alter CYP17A1 or AKR1C3 mRNA expression in PCOS group. In the non-PCOS, AKR1C3 significantly increased with gradual increase in insulin concentrations, 1nM/l (P=0.001), 10 nM/l (P=0.004), and 100 nM/l (P=0.0003). Insulin up regulates AKR1C3 mRNA expression (no treatment (0), 1, 10,100) (0.96±0.21) (1.59±0.84) (2.39±1.23) and (7.42±0.85) respectively. LH± insulin did not alter the expression of either of the enzymes in PCOS

Insulin increased testosterone concentration in non-PCOS but not in the PCOS, testosterone concentration in the supernatant of untreated cultured PCOS (n=5) adipocytes (mean± SEM, 129.3±2.5 pg /ml) was significantly higher ($P<0.0001$) than that (33.7±4.6 pg /ml) of non-PCOS (n=5) adipocytes.

Insulin addition in different concentrations (1nM/l, 10nM/l, 100nM/l) resulted in a significant increase in testosterone concentrations (94.1±7.1; 118.2±18.2, 200.0±7.3 pg/ml, respectively) in the supernatant of cultured non-PCOS adipocytes, but not in the PCOS adipocytes (118.1±1.8, 90.5±6.4, 89.3±7.6 pg/ml, respectively). The increase of testosterone levels in the non-PCOS adipocyte culture supernatant followed a dose dependent fashion. The magnitude of increase in testosterone concentrations in non-PCOS adipocyte culture supernatant was markedly increased when LH was added to insulin. Adding PI3-K inhibitor (LY294002, 10ng/ml) to insulin did not change the magnitude of insulin effects on testosterone concentrations in non-PCOS adipocyte culture supernatant.

Conclusion:

The data obtained suggests that adipose tissue has the ability to produce its own steroid hormone after detection of the main key enzymes of androgen biosynthesis, and it revealed a 5-fold increase in the expression level of AKR1C3 mRNA in subcutaneous adipose tissue of PCOS women. It is therefore possible to postulate that peripheral adipose tissue plays an important role as a source of excess androgen production in women with PCOS. This could potentially pave the way for the development of innovative therapeutic targets for the management of this very common syndrome.

In addition, we show a markedly higher CYP17A1, AKR1C3 and testosterone in peripheral adipose tissue of PCOS vs. non-PCOS women. This supports the hypothesis that peripheral adipose tissue plays an important role in the pathogenesis of hyperandrogenaemia and PCOS. Insulin and LH seem to play a role in the increased androgen synthesis in adipose tissue of PCOS women, but not through the PI3-K signaling pathway. PI3-K signaling pathway does not seem to be involved in insulin regulation of testosterone synthesis in peripheral adipose tissue of women with or without PCOS.

ABBREVIATIONS

AES	Androgen Excess and PCOS Society
ASRM	American Society for Reproductive Medicine
BCA	Bicinchronic acid assay
BSA	Bovine serum albumin
BMI	Body mass index
CAD	Coronary artery disease
Ct	Cycle threshold
DHT	Dihydrotestosterone
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
ESHRE	European Society for Human Reproduction and Embryology
FSH	Follicular stimulating hormone
GLUT 4	Glucose 4 adipose-specific transporter
HBSS	Hanks Buffer Salt Solution
HCL	Hydrochloric acid
HCG	Human chorionic gonadotropin
HDL	High density lipoprotein
HPA	Hypothalamus-pituitary-adrenal
HPO	Hypothalamus-pituitary-ovarian
HSD	Hydroxysteroid dehydrogenase
IDM	Induction differentiation medium
IGF-1	Insulin-like growth factor-1
IGBP-1	Insulin-like growth factor binding protein-1
IL-6	Interleukin 6
IR	Insulin receptor
LDL	Low density lipoprotein
LH	Luteinising hormone
LPL	Lipoprotein lipase
MDM	Maintenance differentiation medium
MAPK	Mitogen activated protein kinase

MB	Methylene blue
mRNA	Messenger ribonucleic acid
NIH	National Institute of Health
NICHD	National Institute of Child Health & Human Development
FNPO	Number of mean follicle per ovary
ns	not significant
OGTT	Oral glucose tolerance test
P450 arom	P450-Aromatase
P450scc	Enzyme P450 side chain cleavage
PBS	Phosphate Buffered Saline
PCOS	Polycystic ovarian syndrome
PDK	Phosphotidylinositide-dependent-Kinase
PFA	Paraformaldehyde
PI3-K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PPAR-Y	Peroxisome proliferator-activated Y
PROG	17-Hydroxyprogesterone
RBP4	Retinol binding protein 4
RTase	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
qRT-PCR	Quantitative real time polymerase chain reaction
SAT	Subcutaneous adipose tissue
SC	Subcutaneous
SD	Standard deviation
SDS.PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SHBG	Sex hormone binding globulin
StAR	Steroidogenic acute regulatory protein
T2DM	Type 2 diabetes mellitus
TBE	Tris/Borate/EDTA
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline+Tween
TNF-α	Tumour necrosis factor alpha
VAT	Visceral adipose tissue
Zn2+	Zinc ion

ZnC12	Zinc chloride

CHAPTER 1 INTRODUCTION

1.1 Polycystic ovarian syndrome (PCOS)

Polycystic ovarian syndrome (PCOS) is the most common endocrine, reproductive, metabolic, and psychological disorder among women of reproductive age (Ehrmann, Barnes et al. 1995).

Ovarian dysfunction is the most common feature of the syndrome. It includes androgen excess, chronic anovulation associated with menstrual disturbance, and infertility (Escobar-Morreale and Millán 2007); (Nardo 2009; Jonard and Dewailly, 2004); (Azziz, Carmina et al. 2009). Moreover, polycystic ovaries (PCO) result from arrested growth of several small antral follicles and hypertrophy of the theca cell layer (Escobar-Morreale and Millán 2007); (Azziz, Carmina et al. 2009); (Dewailly, Robert et al. 1994). In addition to these features, PCOS places women at high risk of developing complications later in life, such as Type 2 diabetes mellitus (T2DM).

1.2 History, definition, and prevalence.

In 1935, Stein and Leventhal first described PCOS, and observed the association between amenorrhoea, hirsutism and PCO (Azziz and Adashi 2015); Stein and Leventhal 1935). However, the underlying mechanism of PCOS remains vague, and until now, there is no clear cause. Indeed, it is a complex disease, and its definition remains subject to controversy and debate (Dunaif and Thomas 2001)

In order to diagnose patients with multiple manifestations of PCOS, several experts have attempted to find diagnostic criteria for PCOS. Three groups of diagnostic criteria have been proposed, reflecting the heterogeneity and complexity of the syndrome, these criteria are summarized in **tab 1.1**.

NICHD/NIH Definition, 1990 Less inclusive 1 and 2 needs to be met:	**Rotterdam Definition, 2004** More inclusive 2 of 3 need to be made:	**AES Definition, 2009**
1. Hyperandrogenism -Clinical (hirsutism, acne, frontal balding) -biochemical (high serum androgen concentration) 2.Menstrual irregularity -chronic anovulation -oligomenrrhoea,>35d	**1.Hyperandrogenism** -clinical or biochemical 2.Menstrual irregularity 3-**polycystic ovaries** (Key difference from NIH)	**1.**Hyperandrogenism**** **(**The main criterion): hirsutism and/or hyperandrogenemia. 2- Ovarian dysfunction: Oligo-ovulation and/or polycystic ovaries. 3-Exclusion other androgen excess or related disorders**.**

Table 1.1 Definitions of PCOS 1990-2009. NICHD, National Institute of Child Health and Human Development; ESHRE/ASRM, European Society of Human Reproduction and Endocrinology /American Society for Reproductive Medicine (ESHRE/ASRM); AES, Androgen Excess-PCOS Society. PCOS, Poly Cystic Ovarian Syndrome.

The National Institute of Child Health and Human Development (NICHD) Conference in 1990 was the first attempt to define PCOS, and included hyperandrogenism and ovulatory dysfunction (Zawadzki and Dunaif 1992).

The Rotterdam criteria were the outcome of a European Society of Human Reproduction and Endocrinology /American Society for Reproductive Medicine (ESHRE/ASRM)-sponsored PCOS Consensus workshop group in 2003, which expanded the definition of PCOS by involving, in addition to hyperandrogenism, ovarian dysfunction, PCO morphology and the requirement for at least two of the three diagnostic features to be present with exclusion of other conditions and related disorders, such as congenital adrenal hyperplasia (CAH) , Cushing's diseases, hyperprolactinaemia (Rotterdam, 2004). However, it is argued that the expanded Rotterdam criteria can result in an over diagnosis or misdiagnosis of PCOS; more significantly, these different phenotypes may not have similar risks of long-term metabolic morbidities.

The Androgen Excess-PCOS Society (AES) proposed new diagnostic criteria in 2009, including androgen excess as the main criterion, and excluded women with ovulatory dysfunction and non-hyperandrogenism, which are included in the Rotterdam criteria. In addition, other causes of excess androgens, such as Adrenal congenital hyperandrogenaemia (ACH) were excluded in diagnosing PCOS (Azziz, Carmina et al. 2009).

Although there are consistencies between the criteria presented by the above three groups, important variances remain. Every group considers PCOS a diagnosis of exclusion, and other diagnoses, such as Cushing syndrome, androgen secreting tumour etc., **see tab 1.2,** must be excluded.

Diagnosis	Causes	Clinical features
Congenital adrenal hyperplasia(non-classical)	Nutrition, over-exercising, chronic disease	Hirsutism, menstrual irregularity, elevation of DHEAS, androstenedione, and testosterone
Cushing syndrome	Hyperadrenalism: Cushing disease represents the presence of an ACTH hypersecreting, pituitary secreting adenoma	Facial and truncal fat accumulation, amenorrhoea, excessive hair growth in sideburn region
Androgen secreting tumour	Ovarian, adrenal	
Hyperprolactinemia	Disruption of the normal pulsatility of an GnRH, consequently, FSH and LH	Oligomenorrhea, amenorrhea, galactorrhea, or infertility
Hypothyroidism	Hashimoto ‘s thyroiditis, inflammation of thyroid	Fatigue, weakness, coarse dry hair, hair loss, muscle cramps, constipation, weight gain, depression, irritability, memory loss, decreased libido, abnormal menstrual cycle

Table 1.2 shows other disorders which have been excluded to diagnose PCOS. DHEAS, Dehydroepiandrostenedione sulphate. GnRH, Gonadotrophin releasing hormone. FSH, Follicle stimulating hormone. LH, Luteinizing hormone. ACTH, Adrenocorticotrophic hormone

In 2006, Azziz found that 20-30% of normal women have evidence of multiple cysts on their ovaries (Azziz 2006). By any group the presence of only polycystic ovaries (PCO) was not considered sufficient to define the syndrome. The NIH/NICHD and the Androgen Excess Society need that patients have signs or symptoms of hyperandrogenism such as hirsutism and acne, or hyperandrogenemia, which is defined as raised free testosterone, decreased SHBG (sex hormone-binding globulin), raised dehydroepiandrosterone sulfate (Azziz 2006). However, ESHER/ASRM (Rotterdam) criteria allows for the diagnosis of the syndrome without the finding of excess androgen. Women with dysfunction of ovulation and the presence of polycystic ovaries are diagnosed with PCOS by the Rotterdam criteria. Another main difference between the criteria is how oligomenorrhea or amenorrhea are observed. The Rotterdam criteria did not need irregular menses or ovulatory disturbance for diagnosis considering that women with regular menstrual cycles could be diagnosed as PCOS in the finding of PCO and hyperandrogenemia or hyperandrogenism (Rottergam 2004). In women with regular menstrual bleeding, subclinical ovulatory dysfunction can occur (Azziz 2006). However, the diagnosis of PCOS in women with regular menses is excluded by NIH/NICHD.

Diagnostic criteria of the Rotterdam and AES to be used to diagnosis PCOS require a reliable method to describe the morphology of PCOS. The morphology of the polycystic ovaries which has been proposed by the Rotterdam consensus group requires the presence of 12 or more follicles measuring between 2 and 9mm in diameter and/or an increase in the volume of the ovaries of more than $10cm^3$; the presence of these features in one ovary is sufficient to define polycystic ovary (Rotterdam 2004). However, since that time, progress in ultrasound imaging has been made, improving resolution and enabling the detection of smaller follicles (Lujan 2013)

The advancement in the technology of ultrasound has emerged the requirement for revisiting the criteria which are used to define the morphology of polycystic ovaries (Lujan 2013) (Allemand 2006). In 2006, Allemand used three-dimensional transvaginal ultrasound in 10 patients diagnosed with PCOS and 29 normoandrogenic ovulatory control to measure the number of mean follicle per ovary (FNPO) and the maximum number of follicles in a single

sonographic plane. A mean FNPO of ≥20.1 detected PCO with 100% specificity and 70% sensitivity, in a single sonographic plane, the maximum number of follicles was 10 and identified PCOS with 100% specificity and 90% sensitivity, by using two-dimensional ultrasound, ovarian volume ≥13.0 cm^3 predicted PCO with 100% specificity and 50% sensitivity (Allemand 2006). In 2011, Dewailly et al the total number of all follicles which were less than 10nm in diameter measured throughout the ovary and the ovarian volume by using two-dimensional transvaginal ultrasound. A threshold follicle number of 19 had a sensitivity to detect PCOS of 81% and a specificity of 92%, ovarian volume of 7cm^3 detected PCO with 87% sensitivity and 89% specificity (Dewailly 2011). In 2013, Lujan et al found that a FNPO threshold of 26 follicles has 85% sensitivity and 94% specificity in distinguishing between subjects with PCOS and controls. For follicle counts in a single section a threshold of nine follicles had a sensitivity of 69% and specificity of 69%. Ovarian volume threshold of 10cm^3 gave a sensitivity of 81% and a specificity of 84% (Lujan, 2013).

The prevalence of the syndrome has not been definitively determined. In fact, it is difficult to give a precise prevalence, because of the heterogeneity of the disorder, and the difference in its manifestation among the population. However, based on the definition of PCOS, according to the NIH/NICHD criteria, it is affecting 4%–8% of women of reproductive age (Azziz 2004). For the variation in the prevalence of PCOS based on the diagnostic criteria used see **tab 1.3**. In 2010, March et al and in 2012, Yildiz et al consistently reported that by using the Rotterdam criteria the prevalence of PCOS is two to three times more than those obtained using the NIH/NICHD.

Reference	Population	NIH/NICHD criteria	ESHRE/ASRM criteria	AES criteria
March et al 2010	728 Australian women	8.7%	17.8%	12%
Mehrabian et al 2011	820 Iranian women	7%	15.2%	7.92%
Yildiz et al 2012	392 Turkish women	6.1%	19.9%	15.3%

Table 1.3 Variations in the prevalence of PCOS based on the diagnostic criteria used. NIH/NICHD, National Institutes of Health/National Institute of Child Health and Human Disease; ESHRE/ASRM, European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine; AES, Androgen Excess and PCOS Society.

1.3 Pathophysiology of PCOS

PCOS is a heterogeneous collection of symptoms and signs which together form a spectrum of a complex disorder with a mild presentation in some, and a severe disturbance of reproductive, endocrine and metabolic function in others (Balen 2004). Its pathophysiology appears to be polyprismatic and investigation of the spectrum of phenotypes from a single approach of pathogenetic view cannot adequately interpret and explained. Environmental and genetic factors are interconnected. Predisposing factors causing multiple aberrations in steroidogenesis, folliculogenesis, and metabolic pathways are continuously identified, however, its main pathogenesis is still not understood well (Diamanti-Kandarakis, 2006)

Two important hormones are identified as having a role in PCOS: androgens and insulin **fig 1.1,** (Nestler, Jakubowicz et al. 1998); (Sharpless 2003)**.** However, the underlying mechanism, which links these two hormones and their association with PCOS, remains unclear.

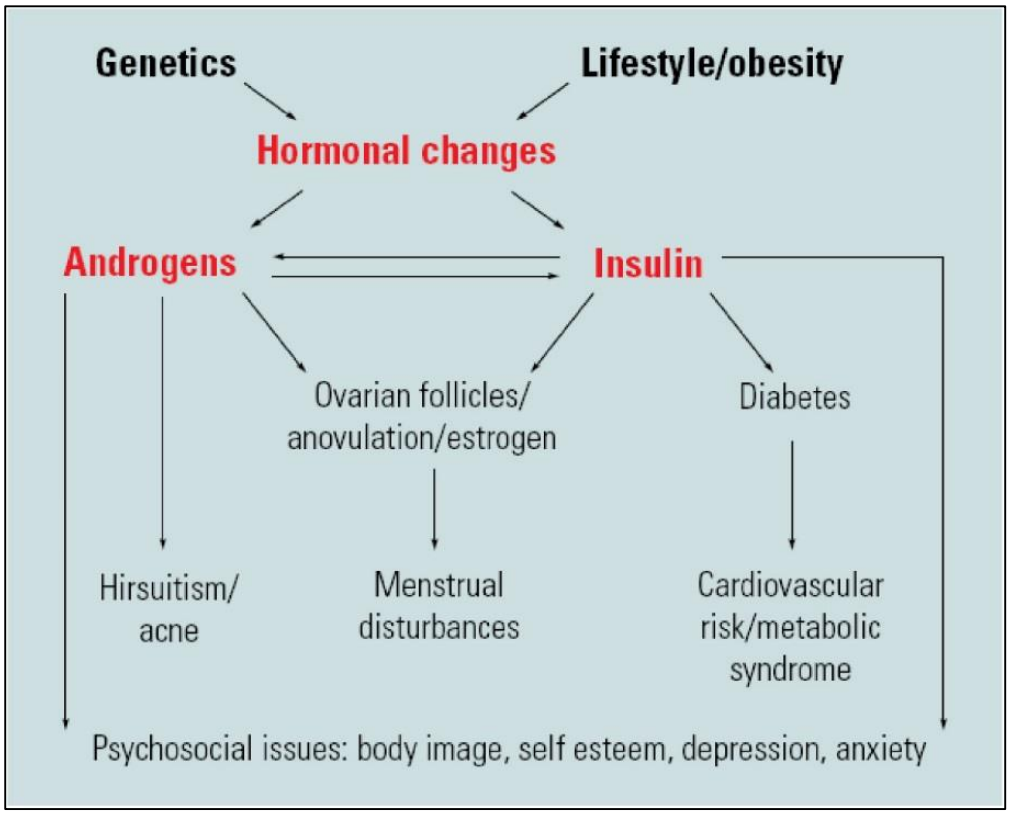


Fig 1.1 Aetiology and clinical features including reproductive, metabolic and psychosocial features of polycystic ovary syndrome (PCOS). Adapted from (Teede 2008).

Environmental risk factors:

The increase in the prevalence of PCOS in populations where the gene pool has been relatively constant confirms that environmental factors are assuming a significant role. In susceptible individuals, the development of obesity is connected to the development of PCOS. In developed countries the modern living environment is characterized by low daily energy consumption and high food supply resulting in common positive energy balance. However, this observation is too simplistic and it appears that there are other unrecognized or poorly understood environmental factors, such as exposure to environmental toxin might also have a role in PCOS (Diamanti-Kandarakis 2006).

The two major components of the syndrome, hyperandrogenemic anovulation and insulin resistance could be effected by the interaction of

environmental factors with a PCOS genetic background. Oligo-ovulation is concomitant with increased obesity and associated hyperinsulinemia. Consequently, hyperinsulinemia with high levels of LH has a negative effect on preovulatory follicles, cessation the differentiation of granulosa cells and leading to premature termination of the ovulatory process (Franks 1998) (Jonard 2004). Among PCOS sisters there are interesting findings exploring the role of obesity. In affected PCOS sisters, body weight might be difference (Legro, 1998). Sisters with irregular cycles and hyperandrogenemia are heavier than those with regular cycles and hyperandrogenemia, whereas unaffected sisters have a lower body weight than affected one (Legro, 2002). In 2002, Taylor found that normal weight women with PCOS eat less than normal women of similar body weight (Taylor 2002).

The quality and the type of food not only the quantity of food may change the phenotype of PCOS, this may be related to different genetic backgrounds. In 2003, Carmina showed that body mass was significantly higher in USA women in comparison with Italian women. However, the dietary constituents and total caloric intake were similar, except for the higher saturated fat components in the diet of USA women. Accordingly, it was hypothesized that the difference in body mass does not relate to diet alone, as their food varied only in the quality of consumed fat and not in the quantity of food. From these findings it was concluded that the difference in body weight in PCOS is related to genetic and lifestyle factors (Carmina, 2003).

Medication is an example of an environmental factor which is implicated in the development of a PCOS phenotype. One of the medications which is widely used is valporic acid, which is a short-chained fatty acid used in the treatment of epilepsy, bipolar disorders as well as migraines and general mood disorders. There is evidence to suggest that women treated with valporic acid may develop stigmata of PCOS, including polycystic ovaries, hyperandrogenism, obesity, and an ovulation, and these features may reverse with stopping of the medication (Franks 2001). Although this is a big argumentative area and there may be clear ethnic variations in susceptibility, there are studies suggesting an increase in weight on this treatment is necessary for the development of PCOS (Diamanti-Kandarakis 2006)

Genetic factors:

Several studies have proposed that genetic factors have a role in the pathophysiology of PCOS (Franks 2006). However, no studies established that a single gene as a significant factor in the mechanisms of the syndrome development. In addition, the mode of inheritance is still unclear.

It is considered that genetic factors play a significant role in the pathophysiology of this syndrome, by setting the stage in ovarian tissue for abnormally high androgen synthesis. The most believed model suggests a probable Mendelian pattern of inheritance where key genetic defects would be transmitted to offspring in a dominant autosomal pattern, with widely different penetrance, based on multiple environmental and epigenetic factors, such as exposure to high levels of androgen during the in utero period (Urbanek 2007). Mutations in the genes of the androgen receptor, sex hormone binding globulin (SHBG), and steroidogenic enzymes may be particularly significant in predisposing to the development of high androgen in PCOS (Abbott, 2002)

Genetic causes in the pathogenesis of PCOS have been suggested in the familial PCOS (Prapas, Karkanaki et al. 2009). Dysregulation in CYP17 might be the result of genetic disorder, but the evidence to support the genetic basis of hyperactivity of this gene is limited (Qin and Rosenfield 1998)

1.3.1 Ovarian impairment

1.3.1.1 Neuroendocrine defects (hypothalamic pituitary ovarian axis defects)

The pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinising hormone (LH), have a central role in reproductive function. Their production and secretion is directly stimulated by hypothalamic gonadotropin-releasing hormone (GnRH) and is also affected by integrated feedback mechanisms with ovarian hormones, oestrogen and progesterone (Azziz, Carmina et al. 2009); (Azziz, Carmina et al. 2006) ; (Taylor, McCourt et al. 1997).

Production of GnRH varies in the menstrual cycle, which is disturbed in PCOS (Belchetz, Plant et al. 1978), and is based on the phases of the menstrual cycle. In the follicular phase, pulsatile release of GnRH from the hypothalamus stimulates the production of FSH from the anterior pituitary. FSH promotes follicular development in the ovary, which leads to recruitment of a dominant follicle, and enhances granulosa cells to form oestrogen from androgen by stimulating aromatase enzymes. An increase in oestrogen level has a negative feedback effect on the hypothalamic-pituitary-ovarian (HPO) axis to stop further FSH production. LH promotes androgen production from theca cells in the follicular phase of the menstrual cycle, and is responsible for initiating oocyte maturation at mid-cycle, ovulation time.

At this time, hypothalamic GnRH pulsatility changes due to increasing oestrogen (positive feedback), which leads to a LH surge that occurs 36 hours before ovulation.

After ovulation, the follicle collapses to form the corpus luteum, which produces oestrogen and progesterone. Both hormones act on endometrium to induce anatomical and functional changes in the glands, blood vessels and endometrial storm. This change occurs in a simplified form in phases: proliferative and luteal or secretory phases. In the absence of pregnancy, after 14 days, the corpus luteum undergoes involution, which leads to a rapid fall in progesterone and oestrogen, initiating menstruation (Owen 1975).

In women with PCOS, dysregulation in the HPO, leads to abnormal growth and maturation of the follicles, subsequent oligo /or anovulation, and an elevation in the LH/FSH ratio (Blank, McCartney et al. 2006). An increase in LH stimulates ovarian theca cells to produce more androgen, whereas the relative FSH deficiency results in disturbances in follicular development. This dysregulation could be related to the change in the pulsatile nature of the secretion of GnRH (Taylor, McCourt et al. 1997).

Hormones	Source	Main function
Gonadotropin releasing hormone (GnRH)	Hypothalamus	Stimulates FSH and LH production from anterior pituitary.
Luteinising hormone (LH)	Anterior pituitary	Triggering ovulation and enhances androgen production.
Follicle stimulating hormone (FSH)	Anterior pituitary	Development of ova and stimulates oestrogen production by stimulating aromatase enzyme.
Oestrogen	Ovaries	Reproduction and sexual development, growth of endometrium.
Progesterone	Ovaries	Steroid hormone involved in the menstrual cycle.
Androgen	Ovaries, adrenal gland, and adipose tissue.	Sexual development and has regulatory function in liver and kidney.

Table 1.4 Hormones of the menstrual cycle

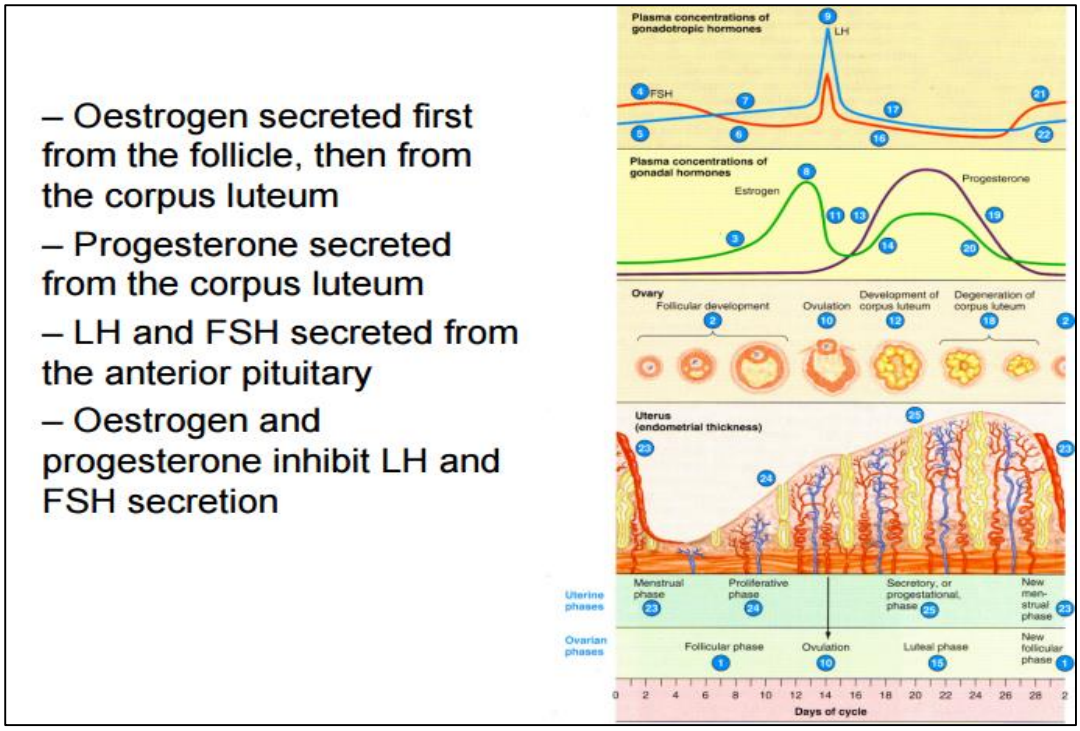


Figure 1.2 Phases of the menstrual cycle. This chart shows the follicular and luteal phases of the cycle, and differences in levels of key hormones required in the development of the ovarian follicle. It also demonstrates the relationship between GnRH frequency, gonadotropic hormone levels and ovarian hormone level during menstrual cycle.

1.3.1.2 Ovarian folliculogenesis

Ovaries are the main reproductive organ in the female, and are essential to fertility in their production of ova and in hormone synthesis. In normal women, small antral follicles grow into large pre-ovulatory follicles, which are selected for ovulation (Escobar-Morreale and Millán 2007); (Jonard and Dewailly 2004). However, in PCOS, there is arrested development of the small antral follicles at an early stage of maturation, which corresponds with the stage of selection of the dominant follicle for ovulation (Franks, Stark et al. 2008). The morphology of ovaries in PCOS is characterised by enlargement with hypertrophied stroma, and a high number of small antral follicles distributed among the periphery of the ovaries under the thick capsule. Dysfunction of

ovulation in PCOS women results from impairment in the development of the mature follicle (Azziz, Sanchez et al. 2004).

The main postulated reason for impairment in the maturation and selection of the dominant follicle is the abnormal endocrine ovarian environment, especially hypersecretion of LH, androgen, and insulin, in the presence of a relative decrease in FSH (Jonard and Dewailly 2004); (Nisenblat and Norman 2009). There is evidence confirming that hyperandrogenism may be involved in the arrest of follicle maturation in PCOS women (Azziz, Carmina et al. 2006). Studying the exposure to androgen for a short time during the ante-natal period in the Rh monkey revealed that there is arrest in the growth of early follicles into pre-antral and small antral follicles (Vendola, Zhou et al. 1998).

1.3.1.3 Ovarian steroidogenesis

Ovaries are the main source of excess androgens in PCOS. The main cause of increasing ovarian androgen production is a disturbance in the LH/FSH ratio. Based on the two-cell-gonadotrophin hypothesis, changes in the LH/FSH ratio can lead to an imbalance between the action of LH in the theca cells in androgen synthesis and FSH in the formation of oestrogen by aromatisation of androgen in the granulosa cell. LH stimulates ovarian theca cells to produce androstenedione; the granulose cells of the growing follicle convert it to oestrogen under the influence of FSH dependent aromatase (Balen 2004).

The amount of androgen production is determined by the number and capacity of ovarian theca cells. In PCOS, the theca cell number is increased and is associated with a rise in the activity of the key enzymes of androgen biosynthesis, resulting in elevated androgen production (Nelson, Legro et al. 1999).

1.3.1.3.1 Ovarian steroidogenic enzymes and biochemical action

It is of fundamental importance to understand the physiology of steroidogenesis, in order to understand the most common complex endocrine disorder in women, namely PCOS.

Steroidogenesis is a process by which cholesterol is converted to biologically active substances known as steroid hormones (Miller 2008). All steroid hormones are formed from the same precursor, cholesterol. Therefore, their structures are closely related, and characterised by the presence of a ring-system structure, which is stable (Miller and Auchus 2011). The general information about the pathway of this process and the relationship between precursor and end product has been explained (Miller 2008).

While most endocrine subjects describe ovarian, adrenal, testicular, placental and other steroidogenic processes in a gland-specific mode, it is better to understand steroidogenesis generally. This process is based on the various enzymes involved and the genes that encode them (Miller and Auchus 2011). Steroidogenesis is catalysed by the same enzyme in all tissues (Miller et al., 2011). For instance, the conversion of cholesterol to pregnenolone (**fig 1.2**) is the first and rate-limiting step in the process of steroid hormone synthesis; it is catalysed by a single enzyme known as cytochrome P450 side-chain cleavage enzyme, P450scc (CYP11A1). It is a complicated step, subject to regulation by several factors, where it also self-regulates the final quantity of the process, known as the quantitative regulation step (Miller and Auchus 2011). One of these factors is the trophic hormonal system which is specific for the type of cell, in each steroidogenic cell. Tropic (peptide) hormones control the overall rate of steroid hormone production (Marsh, 1976). Steroid hormone type which can be produced by a certain type of cells is stimulated by its complement of peptide hormone receptor, its response to the stimulation by peptide hormone and its genetically expressed complement of steroidogenic enzymes**.** In ovarian granulosa cells, follicle-stimulating hormone (FSH) regulates progesterone and estrogen synthesis, whereas in luteinized ovarian granulosa-luteal cells luteinizing hormone (LH) controls progesterone synthesis, androgen production and testosterone synthesis. In adrenocortical fasciculata-reticularis cells, adrenocorticotropic hormone (ACTH) stimulates cortisol/corticosterone and angiotensin II (AII); and in adrenal glomerulosa cells, potassium regulates aldosterone synthesis (Moyle 1973).

On the other hand, qualitative regulation steps are ones that determine the types of steroid to be produced and catalysed by several other enzymes. For

example, testosterone is formed from androstenedione by the action of 17 β-hydroxysteroid dehydrogenase (AKR1C3), whereas oestrogen is formed from testosterone by P450 aromatase (Miller and Auchus 2011).

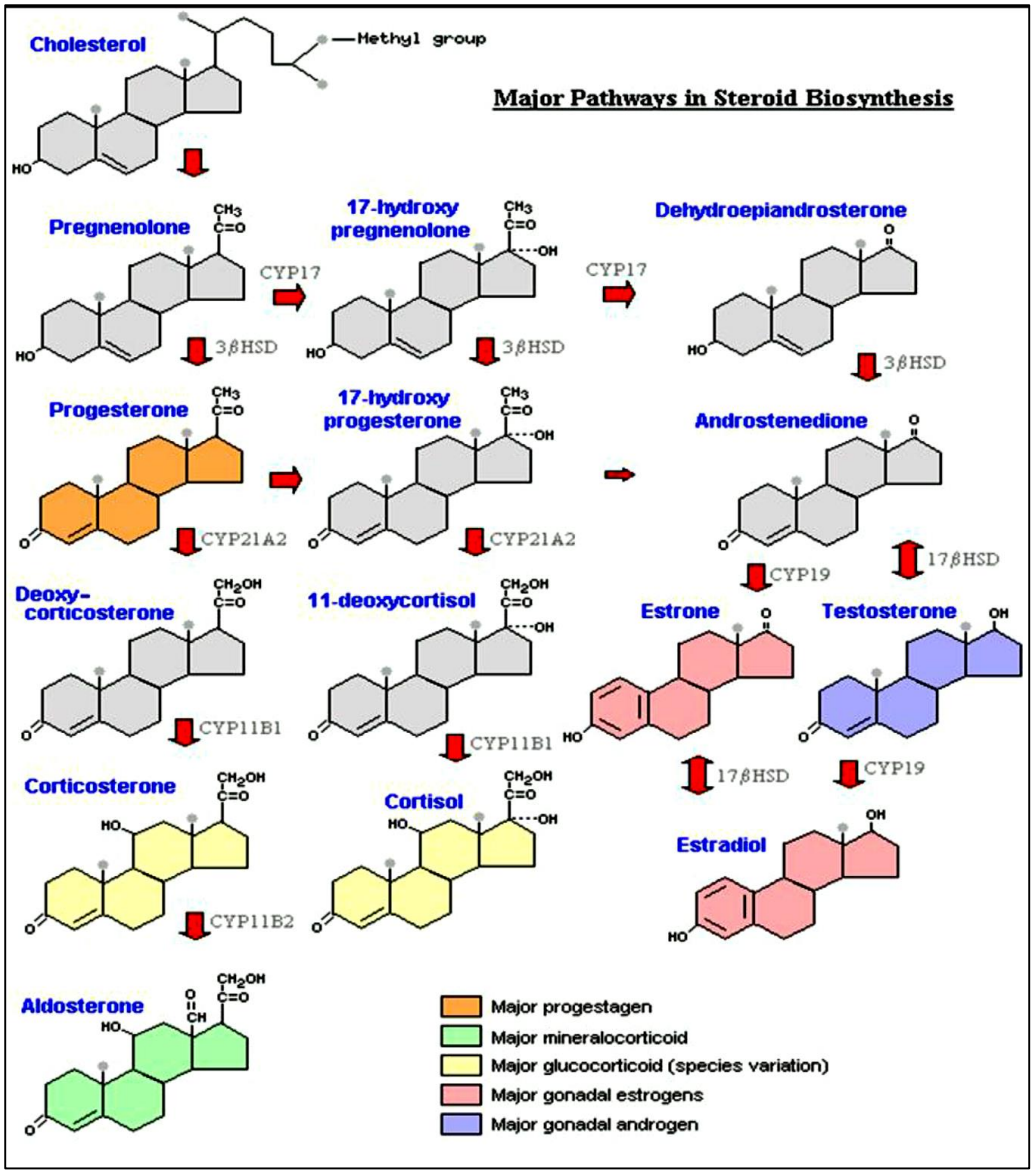


Figure 1.3 shows the major pathways of the steroidogenesis process. CYP17A1, 17, 20 lyase. CYP19, aromatase. (3 β HSD) 3β- hydroxysteroid dehydrogenase. 17β-HSD, 17β- hydroxysteroid dehydrogenase. CYP21A2, 21-hydroxylase.CYP11B2,11 β-hydroxylase. Chart adapted from (Bowen 2010)

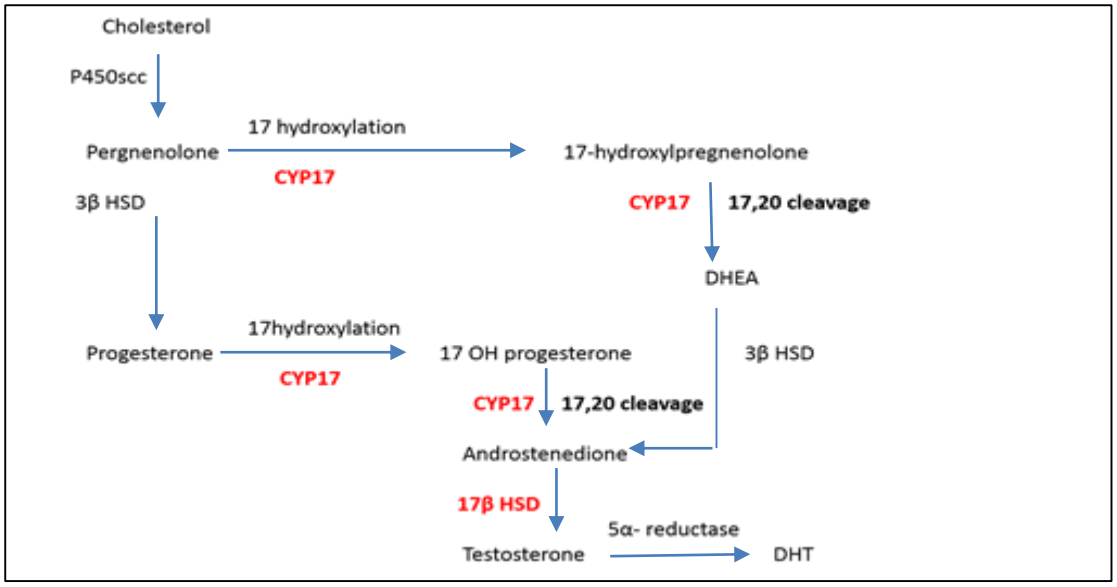


Figure 1.4 Schema of androgen biosynthesis. P450 scc, cytochrome side chain cleavage catalyses, the rate limiting step which is to convert cholesterol to pregnenolone; CYP17, 17α hydroxylase.17, 20-lyase, catalyses the cleavage of the C17-C20 bond of pregnenolone and progesterone to form DHEA, dehydroepiandrosterone, and androsterone respectively; 3β-HSD, 3 beta hydroxysteroid dehydrogenase: 17β-HSD, 17 beta hydroxysteroid dehydrogenase. DHT, dihydrotestosterone.

1.3.1.3.2 Enzymes involved in steroidogenesis

Steroidogenic enzymes are enzymes involved in the biosynthesis of sex hormones, not only in the gonads, but also in peripheral tissues, such as adipose tissue (Luu-The and Labrie 2010). Numerous steroidogenic enzymes with steroid-converting activities have been determined in fat tissue (Blouin, Richard et al. 2003).

Locally, production of sex steroids by peripheral tissue was established by the cloning of multiple types of steroidogenic enzymes, including type 1 and 2 of 3β-HSD (Lachance, Luu-The et al. 1991), types 1,2,3,5, and type 7 of 17β-HSD (Rheault, Dufort et al. 1999), as well as type 1 and 2 of 5α-reductase (Luu-The 2001). The expression of these enzymes in adipose tissue is potentially

important in the local synthesis and inter-conversion of androgen, and in exaggeration of the symptoms and signs of PCOS with obesity (Luu-The 2001).

In general, enzymes in steroidogenesis are divided into two groups: the first group - a cytochrome p450; and a second group - hydroxysteroid dehydrogenase (HSD) (Miller and Auchus 2011).

Cytochrome P450: this group of enzymes is termed P450, because all of these enzymes absorb light at 450nm (Miller and Auchus 2011). Cytochrome P450 side-chain cleavage (P450scc) is a mitochondrial enzyme responsible for the formation of pregnenolone from cholesterol, which works as an intermediate in steroid hormone synthesis. In addition, the core enzyme from the same group is P450c17, which mediates both 17α-hydroxylase and 17, 20-lyase activities (Miller 2008). It has two functions – hydroxylation and by acting as a lyase. The first activity gives hydroxylation of pregnenolone and progesterone at the C17 position to generate 17-hydroxypregnenolone and 17- hydroxyprogesterone, whereas the second enzyme activity cleaves the C17-C20 bond of 17-hydroxypregnenolone and 17-hydroxyprogesterone to form dehydroepiandrosterone and androstenedione respectively (Nakajin 1981) **fig 1.4**. This enzyme is one of those investigated in this study.

Hydroxysteroid dehydrogenase (HSD): This is a group of enzymes involved in steroid biosynthesis and includes 3α-HSD, 3β-HSD and 17β-HSD (Miller and Auchus 2011), which will also be investigated in this research (**fig 1.3**). 17β-HSDs are enzymes that catalyse several reactions: they catalyse the formation of oestradiol (E2) from oestrone (E1); and formation of testosterone from androstenedione (4-dione).

17β-HSD enzymes are widely distributed in human tissues. Their activity is not restricted in the classical steroidogenic tissues, such as ovary (Pittaway 1983), testis (Inano, Ohba et al. 1983), and placenta (Luu-The, Labrie et al. 1990); (Belanger, Luu-The et al. 2002). However, they are also present in large numbers in peripheral intracrine tissue; for example, adipose tissue (Bleau, Roberts et al. 1974), skin, vaginal mucosa and others. There are seven types of human 17β-HSD, and their primary structure have been identified.

The expression of these enzymes is tissue specific and regulated by different factors. There are several findings strongly supporting that peripheral tissue have their own steroidogenic enzymes responsible for independent production of androgen **fig 1.4,** and oestrogens from Dehydroepiandrosterone (DHEA) and/or its sulphate (DHEAS). For example, insufficiency of type 2 3-β-HSD is responsible for congenital adrenal hyperplasia (Lachance, Luu-The et al. 1991); (Labrie, Belanger et al. 1995); (Simard, Durocher et al. 1996).

Type 5 17β-HSD is a steroidogenic enzyme involved in the formation of testosterone from androstenedione (4-dione), while 3α-HSD-3 catalyses the conversion of the active androgen - dihydrotestosterone (DHT) - to inactive metabolites, 5alph-androstan-3alph and 17β-diol (3α-diol). It appears that 17β-HSD-5 is involved in the formation of the active form of androgen, while 3α-HSD are important enzymes in the inactivation of the active form of androgen (Blouin, Richard et al. 2003). The expression of 17β-HSD type 5 is site specific. It was found that its expression in the subcutaneous adipose tissue (SAT) is higher than that in the omentum. This may explain the increase in its androgenicity with increase in Body mass index (BMI) in normal women, and women suffering from PCOS (Dunaif, Segal et al. 1992); (Quinkler, Sinha et al. 2004).

It is involved in androgen production from peripheral tissue, thus, constituting or representing another source of androgen (Luu-The, Labrie et al. 1990) by catalysing the conversion of 4-dione into T (Dufort, Soucy et al. 1996). It belongs to the aldo-keto reductase family. Its activity is highly labile (in the human) and is easily destroyed upon homogenisation of cells or tissue (Khanna, Qin et al. 1995).

Type 5 is found in the theca cells of the ovary, which is responsible for androgen production in women; however, in contrast with type 3 which is not found in the ovary and responsible for pseudohermaphroditism in deficient men. Type 3 deficiency is asymptomatic in women (Geissler, Davis et al. 1994); (Pelletier, Luu-The et al. 1999)(Luu-The, 2001).

1.3.1.3.3 Sex steroid hormones

Testosterone, dehydroepiandrosterone sulfate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione, and androstenediol, are androgens secreted by the endocrine glands. Androstenediol has both androgenic and estrogenic activity. The only androgens with direct androgenic activity are Testosterone and its biologically active metabolite dihydrotestosterone (DHT). DHEAS, DHEA, and androstenedione are all precursors of testosterone (Miller 2010).

Testosterone in the classical main steroidogenic organs, namely ovaries and adrenal gland, can be produced mainly via two pathways: (i) progesterone, 17α-hydroxy progesterone, and androstenedione, or (ii) 17α-hydroxypregnenolone, DHEA, and androstenediol (Pochi 1974). Alternatively, in peripheral organs or tissue, such as adipose tissue, which is the main tissue investigated in this work, testosterone can be produced by conversion of circulating DHEAS through the action of several enzymes; for example, 17β-HSD (Labrie, Simard et al. 1991) (Fritsch 2001). In the cells or tissue, testosterone can be activated to the most potent tissue androgen, 5α-DHT, through the action of 5α-reductase, and can be inactivated to oestradiol by aromatase (Kaufman 1996).

1.3.1.3.4 Hyperandrogenism

Hyperandrogenism refers to supernormal levels of circulating androgens such as total, free testosterone (T), androstenedione, dehydroepiandrosterone (DHEA), DHEA metabolite DHEA sulphate (AHEAS) (Azziz 2009). An excess of androgen level is one of the most frequently occurring symptoms of unexplained hyperandrogenism seeming to arise from dysregulation of ovarian and adrenal androgen secretion (Nelson, Qin et al. 2001). P450c17 (CYP17A1) appears to be the rate-limiting step, and its regulation is described at a different level. CYP17A1 is one enzyme with the activity of 17α-hydroxylase/17, 20-lyase. This is a functional and not genetic or structural distinction (Zuber 1986). It is expressed in gonads and adrenal glands (Chung 1987), and also detected in the adipose tissue (Puch 2002).

17α-hydroxylase converts both pregnenolone and progesterone with the same efficiency (Lee-Robichaud., 1995), but 17,20-lyase activity in the conversion of 17-α hydroxypregnenolone to DHEA is about 50 times more efficient than in the conversion of 17-hydroxyprogesterone (17-PROG) to androstenedione. This is consistent with the large amount of DHEA produced by the adrenal gland in the human adult and foetus (Lee-Rochaud et al. 1995). Therefore, under normal conditions, 17-PROG is not an important substrate in the synthesis of sex steroids in humans (Miller and Auchus 2011).

Clinical data suggest the hyperfunction of CYP17A1 in PCOS. With administration of Gonadotropin releasing hormone agonist (GnRHa) (Ehrmann, Barnes et al. 1995), there was hypersecretion of 17-PROG production without evidence of steroidogenesis blocking.

In addition, they found with administration of dexamethasone to women with PCOS, that there was no suppression of free testosterone to normal levels. However, in women with isolated adrenal hyperandrogenism, the level of testosterone was suppressed with administration of dexamethasone. These observations suggest that the PCOS is due to dysregulation of CYP17 in the ovary, adrenal gland or both organs (Rosenfield, Barnes et al. 1990). However, the basis of the dysregulation is unclear.

In 2006, Blank et al state that "……the concomitant disruption of neuroendocrine function would serve to further exacerbate hyperandrogenaemia…." this was depicted in their schema (Blank 2006). Thus, in their reviews they tend to show that high LH enhances hyperandrogenism by stimulation of ovarian androgenesis. However, this is unlikely to be valid although of the fact that changes in LH secretion in women with PCOS is the main neuroendocrine component, and the high ovarian androgen secretion results from primary LH excess which has long been considered the main cause of high androgen in PCOS (Doi 2008). This concept came from that the stimulatory effect of LH on the function of ovarian theca cell and the elevation of serum LH levels at baseline and in response to gonadotrophin releasing hormone (GnRH) in women with PCOS. Like this role of LH excess is debateable. However, in culture theca cells from polycystic ovaries (PCO)

produce abnormal volumes of steroids, before and after treatment with LH (Gilling-Smith, 1994). In addition, with administration of a fixed dose of the LH analogue human chorionic gonadotropin in patients with PCOS there were hyper-responses of 17-hydroxyprogesterone and androstenedione (Ibanez 1996). These continued to be observed despite adjustment of their baseline LH abnormality by treatment with a GnRH agonist for 1 month (Gilling-Smith 1997). This result led to use of GnRH as a certain test of the combined function of the ovarian follicular compartments. Characteristically, patients with classical PCOS (hyperandrogenism with high LH or ultrasonographic abnormality or both) respond to this test (a GnRH agonist challenge test) with hyper-responsiveness of plasma 17-hydroxyprogesterone and, or a lesser extent, androstenedione (Ehrmann 1995). This form of steroid secretion postulates that there is general dysregulation of ovarian androgen secretion, that is specifically prominent at the level of CYP17 activity (17-hydroxylase and 17, 20-lyase). In normal women who received a single dose of a GnRH agonist, such as nafarelin or leuprolide, have premature surge of LH and FSH that is preovulatory levels (Ehrmann 1995), and elevation in the plasma estradiol and estrone 3-fold within 24 hours.

In women with PCOS, this form of estrogen rising has been observed in more than half of hyperandrogenic patients, several of whom did not have the classic criteria for the diagnosis of PCOS. In 1992, Ehrmann demonstrated this observation in his study, where he found that 58% of hyperandrogenic patients had elevation in plasma 17-hydroxyprogestrone in response to a GnRH agonist.

Androgen Biosynthesis is an intrinsic characteristic of PCOS theca cells, however, increase in the transcription of steroidogenic enzyme genes could come from the constancy of the metabolic imprinting of the theca cells imposed in vivo and maintained in vitro rather than genetic imprinting (Nelson 1999). Furthermore, the sensitivity of PCOS theca cells might be increased due to some content of the culture medium which stimulates steroidogenic enzymes expression (Nelson 1999).

In 2008, Doi concluded that based on evidence, LH is not a primary factor in the development of hyperandrogenism of PCOS, and high LH may be a result of the metabolic changes in PCOS (Doi 2008).

1.3.1.3.5 Regulators of steroidogenesis

In addition to the high level of LH, in the classical PCOS, insulin resistance and compensatory hyperinsulinemia are common. It becomes clear that high levels of insulin have a significant role in the pathogenesis of PCOS via stimulation of ovarian androgen production (Rosenfield, Barnes et al. 1990); (Nestler, Jakubowicz et al. 1998), and by decreasing the concentration of sex hormone binding globulin (Nestler, Powers et al. 1991).

It appears that insulin acts as an endocrine regulator for steroidogenesis by stimulating the activity of CYP17 (Nestler, Jakubowicz et al. 1998). It works through activation of receptor tyrosine kinase (Whitehead 2000), which phosphorylates different protein targets to those in the Protein Kinase A (PKA) transduction signal.

The ability of insulin to work through different signal pathways enables it to augment the stimulatory action of gonadotrophins in ovarian steroidogenesis (Franks, Gharani et al. 1998); (Willis, Watson et al. 1998). In 1993, in an *in vitro* study, (Bergh, Carlsson et al. 1993) found that insulin can augment the action of LH in the stimulation of androstenedione (androgen) production from ovarian theca cells.

In addition to insulin, Insulin growth factor-1 (IGF-1) has a direct relationship with PCOS. It was found that administration of IGF-1 in female patients with Laron syndrome (Klinger 1998) is associated with elevated serum testosterone, androstenedione, and LH/FSH ratio. With reduction in the IGF-1 administered, the level of androgen returned to normal (Nahum 1995). The development of PCOS with the induction of IGF-1 is another suggestion that high androgen levels in PCOS are due to excessive steroidogenesis with abnormality in steroid biosynthesis (Qin 2000).

The role of insulin in ovarian steroidogenesis and folliculogenesis disturbance in PCOS is clear from the above. Therefore, it is important to

describe this regulator in detail, in order to understand the role of insulin in the development of PCOS, particularly hyperandrogenism.

1.3.1.4 Insulin resistance, compensatory hyperinsulinemia and pathophysiology of PCOS

Although insulin resistance affects 50-70% of women with PCOS, it is not included among the diagnostic criteria for PCOS (Azziz 2005). However, it is clear from the above that insulin has a role in ovarian steroidogenesis and disturbances of folliculogenesis.

Insulin is a peptide hormone produced by the β-cells of the pancreas. It maintains normal glucose levels in the blood (4.4 to 6.1 mmol/l) by facilitating cellular glucose uptake, and regulating the metabolism of carbohydrates, and by balancing its secretions and action in target tissues (Garruti 2009). Furthermore, through its mitogenic effects, it promotes cell division and growth (Wilcox 2005).

Insulin mediates its action by binding to receptors on the cell membrane, which consist of two subunits: extracellular (α subunit) and intracellular (β subunit) linked by a disulphide bond (Kido 2001). Conformational change resulting from binding of insulin to the extracellular subunit enables ATP to link to the intracellular subunit (Wolever 1990). In turn, its binding triggers phosphorylation of the intracellular subunit (β subunit), and promotes the activity of tyrosine kinase. This permits phosphorylation of tyrosine in intracellular substrate protein, known as insulin responsive substrates (IRS) (Wolever 1990).

Phosphorylated IRS binds to the signalling molecules mediating the cellular action of insulin (Kido 2001), such as phosphatidylinositol 3-kinase (PI3-K) (Kido 2001), which enhances the metabolic effect of insulin, protein, glycogen, and lipid synthesis (Burks 2001). The other signalling molecules are mitogen adenosine phosphokinase (MAPK), which mediates the mitogenic effect of insulin (Kido 2001).

In addition to insulin, other ligands for insulin receptors are the insulin-like growth factors, which are structural homologs to proinsulin, and have a mitogenic effect (Kim 1997; Hill 1989). Insulin can bind to IGF-1 and IGF-2 receptors, but with lower affinity (Wilcox 2005) **Fig 1.3**

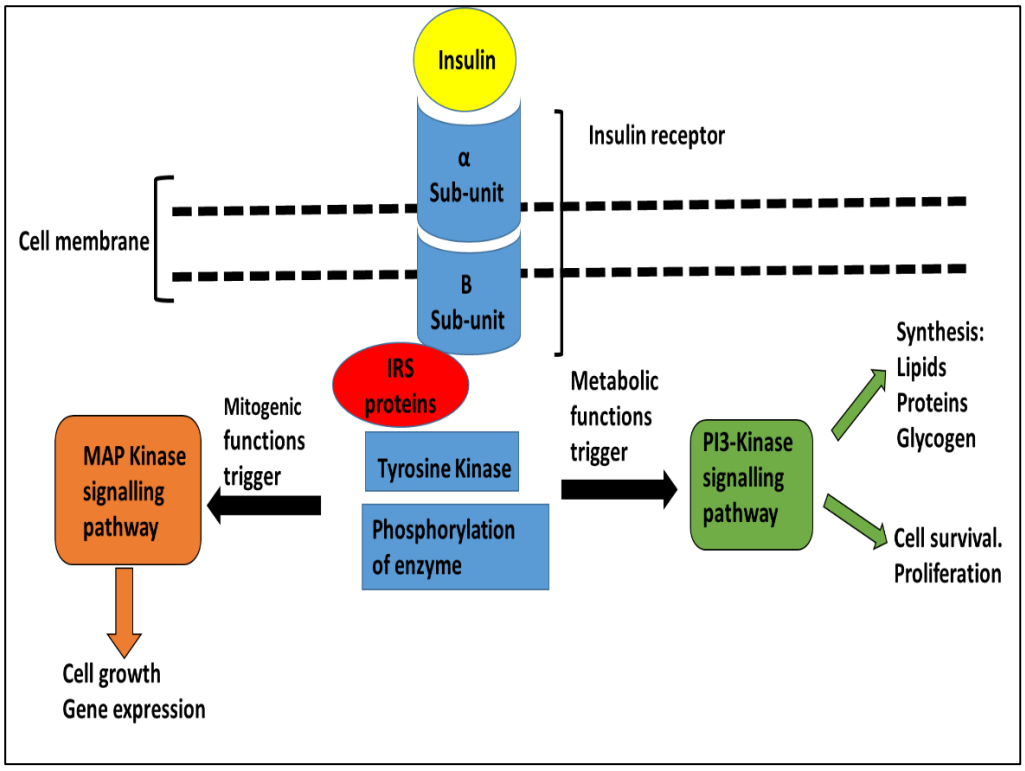


Fig 1.5 Insulin signal pathways. Insulin combines with insulin receptor which is a heterotetramer consisting of two subunits, α and β- subunits linked by disulfide bonds. The α-subunit is the extracellular part containing the ligand-binding site, and the β-subunit is the intracellular part of the insulin receptor containing a ligand-activated tyrosine kinase. Autophosphorylation of tyrosine increases the receptor's tyrosine kinase activity. The tyrosine-phosphorylated insulin receptor phosphorylates intracellular substrates, such as insulin receptor substrate (IRS)-1 and IRS-2, initiating signal transduction and the plieotropic actions of insulin. The stimulation of PI3-K (PI3-kinase) by tyrosine-phosphorylated IRS-1 appears to be the pathway for insulin-mediated glucose transport. The MAP kinase pathway appears to control cell growth and glycogen synthesis (Kahn 1994).

1.3.1.4.1 Insulin resistance

Insulin resistance is a condition defined as a defect in the biological response of target tissues to the physiological amount of insulin (Garruti 2009). Classically, impairment in insulin-mediated glucose disposal is a main hallmark of PCOS (Reaven 2004). In women with PCOS disorder at high risk for metabolic syndrome, and coronary artery disease, insulin resistance may be the first marker of metabolic syndrome (Traub 2011). Compensatory hyperinsulinemia results from the additional secretion of insulin from the pancreas, in order to compensate for insulin resistance and to avoid hyperglycaemia (Garruti 2009). This feature makes women with PCOS at high risk of developing T2DM. It has been reported that 30-40% of women with this complex condition have impaired glucose tolerance, and 10% develop T2DM by the age of 40 (Azziz 2006), including defects in insulin secretion (Ehrmann, 1995) and in insulin action (Dunaif, 1997). In 2012, Morgan et al demonstrated beta cell dysfunction in PCOS in the absence of impairment of glucose tolerance. Burghen (1980) was the first investigator to establish the presence of hyperinsulinemia in women with PCOS.

In 2012, Morgan et al concluded that the major health risk of women with PCOS is type 2 DM, and they found that the magnitude of the risk related to the syndrome is 1.5-to 2 fold in comparison with non-PCOS controls (Morgan, 2012).

70% of women with PCOS are insulin resistance, and 10% of them have DM (Freeman, 2010). In PCOS, the rate of conversation of glucose metabolism from normal to abnormal over just three years is 25% (Pesant 2011). More alarming, in adolescents with PCOS, insulin abnormalities are highly prevalent (Bhattacharya 2010). In 2010, Karakas found that 20% of young Thai women with PCOS have DM. Overall, on an oral glucose tolerance test (OGTT) the normal glucose levels do not detect insulin resistance (IR), although of that normal glucose levels correlate C-reactive protein (CRP), dyslipidaemia and other (coronary artery diseases) CAD risk factors (Karakas, 2010). Therefore, there is deficiency of glucose level sensitivity in the detection of the metabolic

risk in women with PCOS (Traub, 2011). It is likely that precursor conditions of insulin abnormalities could predict long term CAD risk well before abnormalities of glucose levels. Insulin resistance could be just as severe in both diabetics and non-diabetics (Ishihara, 2011) - emphasising the significance of this metabolic disturbance as a precursor and not a separate disorder.

In 2010, Yang et al provided direct evidence of end organ disease by showing that insulin resistance alone damages myocardial cells in animal models (Yang 2010). Dysfunction of left ventricular from human data links to IR (Basyigit, 2010). In 2010, Wang found that in PCOS women with different phenotypes the insulin resistance in response to a 3 h 75g OGTT was the same (Wang, 2010).

Insulin resistance in obese PCOS women in comparison with lean PCOS women tends to be high. Several studies show that the correlation between high androgens and IR is significant, but not as important as the link between insulin abnormalities and obesity (Acien 1999). In 2002, Jayagopal et al observed that there was a greater difference in insulin parameters in PCOS women compared to control, and independent of weight (Jayagopal, 2002). In animal studies, prenatal exposure to testosterone led to downstream insulin resistance in early postnatal life (Padmanabhan, 2010). Some human studies show that there is a high degree of correlation between hyperandrogenism and insulin resistance, and it appears that the relationship between high androgens and insulin resistance is different between PCOS and non-PCOS women (Luque-Ramirez 2010). In PCOS women, dysfunction of reproductive functions may also be a manifestation of insulin resistance. In 2010, Strowitzki et al observed that there was a correlation between irregularity of menstrual cycles and the Homeostasis Model Assessment (HOMA)-insulin resistance (Strowitzki 2010). Difficulties of reproductive function in women with PCOS may be related to molecular defects in insulin action. Though endometrial tissue of women with PCOS morphologically appears similar to non-PCOS and may have a similar prevalence of insulin receptors, the action of insulin receptors on endometrium tissue is impaired and this is reflected by lower pregnancy implantation rates (Fornes 2010).

In 2010, Dickerson found that there was a positive correlation between HOMA-IR and follicle count in women with PCOS, during *in vitro* fertilization (Dickerson, 2010). In 2010, Takikawa observed that follicular insulin levels correlate with the outcome of pregnancy after in vitro fertilization (IVF) (Takikawa 2010). In fact, the mechanisms of insulin reproductive abnormalities are still unclear and not fully understood. The most suggested mechanisms for insulin abnormalities include ovarian steroidogenesis dysregulation, excessive LH secretion and glucose uptake abnormalities (Baptiste 2010). Post-receptor insulin abnormalities as well as reduced peripheral insulin receptor binding have been found in women with PCOS (Marsden, 1994).

Compensatory hyperinsulinemia occurs because of increasing insulin from pancreatic β cells, in the presence of peripheral insulin resistance in the muscle and adipose tissue, in order to maintain normal blood glucose levels (Wilcox 2005).

1.3.1.4.2 Mechanism of insulin resistance

In the vast majority of cases, insulin resistance is believed to arise at the cellular level through a defect in the post receptor signalling of insulin. Although there are experimental *in vitro* findings of defects in post receptor insulin receptor signalling, their relevance to human insulin resistance is still unclear (Wilcox 2005). The suggested mechanisms include down-regulation, deficiencies or genetic polymorphism of tyrosine phosphorylation of the insulin receptor, PI3-K; or might include defects in GLUT 4 protein function (Wheatcroft 2003).

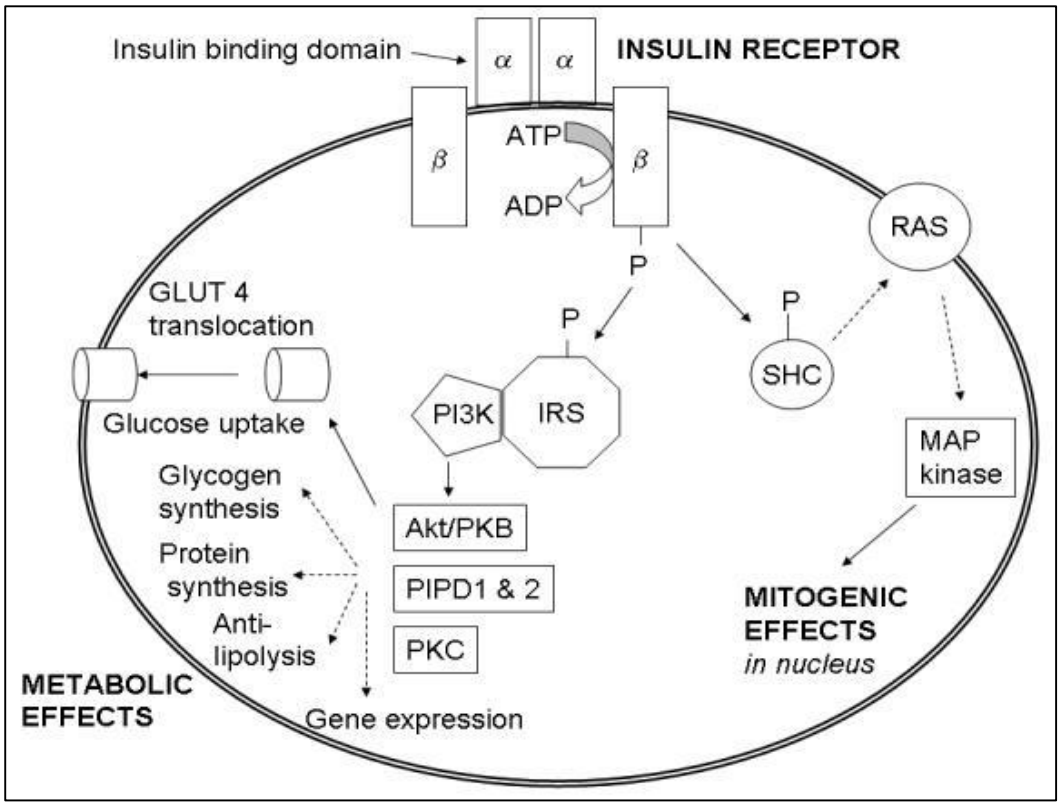


Figure 1.6 shows insulin transduction signalling. Akt/PKB = protein kinase B, GLP-1 = glucagon-like peptide 1,ATP = adenosine triphosphate,IRS = insulin receptor substrate, ADP = adenosine diphosphate, MAP kinase = mitogen activated protein kinase, cAMP= cyclic adenosine monophosphate, PACAP = pituitary adenylate cyclase-activating polypeptide, DAG = diacylglycerol, PI3K = phosphatidylinositol 3-kinase, GIP = gastric inhibitory peptide /, PIPD1 & 2 = phosphatidylinositol dependent protein kinases 1 & 2, glucose-dependent insulinotropic polypeptide, PKC = protein kinase C. Adapted from (Kido 2001)(Hunter, 1998)(Giagulli 1989)

The number and affinity of the insulin receptors appear to be normal in most target tissues of women with PCOS (Dunaif 1996). However, several studies have found that insulin resistance in PCOS occurs due to molecular defects of the insulin receptor. This primarily includes a post-binding defect in the insulin signalling pathway in fat cells and skeletal muscle (Diamanti-Kandarakis and Papavassiliou 2006); Dunaif 1996). The impairment is only in the PI3K signal transduction pathway that mediates the metabolic signalling of insulin. Other signals, such as those that mediate cell growth through the mitogen-activated protein kinase cascades, are preserved (Cusi 2000).

The effect of insulin resistance on tissues varies, and is dependent on the physiological function of the tissue, and on insulin in its metabolic action (Wilcox 2005).

The main target tissues of insulin are skeletal muscle, liver and adipose tissue. Skeletal muscle is the main target tissue for glucose balance, because it accounts for 85% of all the body's insulin-stimulated glucose uptake (James 1994; James and Piper 1994).

Compensatory hyperinsulinemia, which results from selective peripheral insulin resistance as mentioned above, has several complications in acting on the liver, adrenal gland and the pituitary gland. In the liver, it inhibits the production of sex hormone binding globulin (SHBG), resulting in an increase in the fraction of active androgen in the circulation (Yki-Jarvinen 1995; Nestler 1991). In the adrenal gland, it enhances the action of adrenocorticotrophic hormone (ACTH) in androgen adrenal secretion (Moghetti 1996).

In the pituitary gland, based on *in vitro* studies, insulin stimulates the secretion of LH from cultured pituitary rat cells (Adashi 1981; 1990). In humans, based on clinical research, the action of insulin in PCOS on gonadotropin secretion has not yet been detected. However, acute administration of insulin to normal lean women enhanced the LH pulse frequency (Patel 2003).

1.3.1.4.3 Metabolic syndrome and PCOS

The metabolic syndrome (MS) is a cluster of cardiovascular risk factors, consisting of impairment of fasting glucose levels, central obesity, dyslipidaemia and hypertension (Kandaraki 2009). Although the clinical significance of MS is still controversial, based on the fact that the MS may determine a higher risk for cardiovascular diseases than the group of the risks made by its individual components. The common recognized link between its coexisting abnormalities is insulin resistance (Kandaraki 2009).

For clinical and research purposes, the most commonly used definition is a definition proposed by the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATPIII) (Grundy 2004). The most recent criteria of the MS were proposed by the International Diabetes Federation (IDF), which resembles the NCEP definition, with the exclusion that central obesity, evaluated based on ethnicity-specific cut-offs, is an essential part of the IDF definition (Alberti 2005) **tab 1. 4.**

WHO	NCEPATP III	IDF
T2D or IFG or IGT or insulin resistance plus ≥ 2 of the Following: • BMI > 30 kg/m2 or WHR > 0.85 • HDL < 1.0 mmol/L (< 40 mg/dL) • TG ≥ 1.7 mmol/L (150 mg/dL) • BP ≥ 140/90 mmHg or use of blood pressure medication • microalbuminuria > 20 pg /min • Alb/Crea ratio ≥ 30 mg/g	≥ 3 of the following: • WC ≥ 88 cm • HDL < 1.3 mmol/L (< 50 mg/dL) • TG ≥ 1.7 mmol/L (150 mg/dL) • BP ≥ 135/85 mmHg or use of blood pressure medication	Central obesity defined as WC above the ethnicity specific cut-off plus ≥ 2 of the following: • TG ≥ 1.7 mmol/L (150 mg/dL) or specific treatment • HDL < 1.3 mmol/L (< 50 mg/dL) or specific treatment • BP ≥ 135/85 mmHg or use of blood pressure medication • fasting plasma glucose ≥ 5.6 mmol/L (100 mg/dL) or previously diagnosed T2D

Table 1.5 definitions of the metabolic syndrome. The most proposed commonly used diagnostic definitions of the MS. Abbreviation, BP = blood pressure; HDL = high density lipoprotein cholesterol; IGT = impaired glucose tolerance; T2D = type 2 diabetes; TG = triglycerides; WC = waist circumference; WHR = waist to hip ratio. Table adapted from (Kandaraki 2009)

In the general population during the last decade, the prevalence of MS has increased. In women, this increase in the prevalence of this syndrome has been steeper, particularly in the young (Regitz-Zagrosek 2007) (Ramos 2008). The prevalence of MS varies among ethnic groups and age, and also differs according to the definition used for diagnosis. It was found that its prevalence in healthy, reproductive aged women is 26.5% from the National Health and Nutrition Examination Survey (NHANES) (Ramos 2008).
The NCEP ATP III and the IDF definitions of the MS were used in this survey. Although of that the comparative analysis of NHANES data taken from 1988 to 1994 and from 1999 to 2004 showed that the prevalence of MS increased by 8.7% (17.8% to 26.5%), however, in premenopausal females the incidence of MS in comparison with men of the same age is low (Ramos 2008). Therefore, premenopausal women appear to protected from cardiometabolic disorders which seems to be abrogated in women with PCOS (Ramos 2008).

The similarity between MS and PCOS is based in the fact that the definitions of these disorders have produced extreme arguments among experts in the field (Kandaraki 2009). In women with PCOS, the prevalence of MS seems to be significantly higher than the one evaluated in their age matched counterparts from the general population. The relationship between PCOS and MS is not limited to Caucasian women with PCOS, in 2008, Soares and his colleagues documented that in Brazilian, Chinese, Korean, and Indian and in multiracial PCOS populations, the prevalence of MS is high - at least in overweight/ obese patients (Soares 2008) (Dokras 2005).

The prevalence of MS reported from four US studies in predominantly obese women fulfil the NCEP ATPIII criteria for MS and with PCOS is 33.4% to 47.3% (Dokras 2005) (Glueck 2003). However, these prevalence rates to some extent may reflect the effect of the high prevalence of obesity independently of PCOS (Dokras 2005) (Glueck 2003). In addition, three of the above US studies did not include specifically chosen control groups (Glueck 2003) (Ehrmann, 2006).

In 2005, Apridonidze and his college was evaluated the relative increase of MS in women with PCOS by comparing the results of the study to the data acquired from the Third National Health and Nutrition Examination Survey (NHANES III). This method of evaluation should be considered a limitation of this study.

In a European study, in 2006, Carmina reported that the prevalence of MS among PCOS populations with lower BMI is significantly lower than that documented by US studies (Carmina 2006). Although the differences between European and US investigators, some researches have shown that the prevalence of MS in European PCOS patients is higher than in ethnicity-matched controls of similar age (Vural 2005) (Vrbikova, 2005).

However, not only the presence of PCOS produces difference in the study's findings but also the differences in BMI may also contribute to the findings of the studies as none of the above studies have involved patients and controls properly matched for age and BMI (Carmina 2006) (Vrbikova 2005).

In fact, the differences in the rates of MS among different ethnic groups may be related to the variation in the criteria used for PCOS diagnosis. As in studies from the US, the selection of patients was dependent on NIH criteria, which are expected to be more severely metabolically affected than the patients chosen by Rotterdam criteria in European studies (Diamanti-Kandarakis, 2007). Therefore, among phenotypically heterogenous group of women with PCOS diagnosed by Rotterdam criteria, the rate of MS is expected to be low. Accordingly, the prevalence rates of MS are low in women with hyperandrogenism and polycystic ovaries (PCO), but with normal ovulation; as well as in women with anovulation and PCO, but with normal androgen levels; in comparison to the respective rates in women with classic PCOS (Shroff 2007). Most significantly, in the group of PCOS women with anovulation and normoandrogenemic, the prevalence of MS was not significantly higher than the age-BMI matched controls (Shroff 2007).

In addition to the impact of diagnostic definitions of PCOS on the findings of the studies, the criteria used for the diagnosis of MS also has affected. The NCEP-ATPIIII criteria was used in the majority of studies for the diagnosis of MS in PCOS. However, in 2006, Carmina used both the World Health Organization (WHO) and the NCEP ATPIII criteria, and has suspected that the former set of criteria may be more suitable for the evaluation of MS in PCOS women with a lower degree of obesity (Carmina 2006).

This proposal sounds reasonable, since the MS definition by WHO criteria is less focused on obesity and concentrates more on the presence of insulin resistance or glucose intolerance tab 1.4. Consequently, in 2005, Vural revealed that the prevalence of MS diagnosed based on the WHO criteria is significantly higher in PCOS women than in controls, whereas its prevalence with the NCEP criteria is a significantly low, thus enabling to explore any variation between PCOS women and controls (Vural 2005). However, in 2008, Cussons reported comparable rates of MS among a mixed population of obese and non-obese Caucasian PCOS women in which either the NCEP, the WHO or the IDF criteria were applied for MS diagnosis (Cussons 2008).

Several studies documented that most women with PCOS usually have isolated metabolic disturbance, such as lipid abnormalities typical of dyslipidemia, even in the absence of the full blown MS. In 2007, Diamanti-Kandarakis E, observed that 70% of women with PCOS have an abnormal lipid profile (Diamanti-Kandarakis 2008). In 1992, Conway reported that both obese and lean women with PCOS have dyslipidemia. However, in 1997, Rajkhow did not confirm this observation. (Rajkhowa, 1997). In 1992, Conway found that lean PCOS patients display more often an atherogenic lipid profile, such as high levels of total cholesterol and LDL-C as well low levels of HDL-C (Conway 1992). Moreover, women with PCOS display additional risk factors of cardiovascular disease, which are not comprised in the classic definitions of MS such as: low-grade inflammation (Diamanti-Kandarakis 2006), impaired fibrinolysis, increased marker of oxidative stress (González, 2006) and increased levels of Advanced Glycation End products (AGEs) (Diamanti-Kandarakis 2008) - which enhance predisposition to cardiovascular disorders.

The association between PCOS and MS is bilateral. So, not only is MS established among PCOS women, but also women with MS may commonly suffer from reproductive/endocrine features of PCOS (Kandaraki 2009). Reproductive and endocrine abnormalities, which characterize PCOS, commonly associate with obesity, especially visceral fat excess, and insulin resistance (Pasquali 2006). In general, obese women have anovulation (Pasquali 2006). Moreover, increases in the production of potent androgens by adipose tissue has been observed even with simple obesity, especially visceral adiposity (Corbould 2002). In addition, anovulation, hirsutism, and insulin resistance - which are associated with PCOS - have been successfully treated by bariatric surgery. In 2005, Eid found after one year of surgical treatment of that with 24 severely obese (initial mean BMI: 50kg/ m2) young women with PCOS with, Roux-en Y gastric bypass surgery, the return of normal menses and improvement of hirsutism in the studied population paralleled with a decrease in body weight (Eid 2005). In 2005, Escobar-Morreale reported the same results from a small cohort study of morbidly obese women with PCOS treated with bariatric surgery (Escobar-Morreale 2005). In 2001, Korhonen compared the frequency of PCOS features (reproductive/endocrine) in premenopausal women with and without MS, and reported that 46.2% of women with MS had oligomenorrhea in comparison with only 15.1% of lean control and 25.4% of obese control (Korhonen 2001). Moreover, in 2003, Korhonen observed that in women without PCOS but with a hyperandrogenemia - defined by an increased free androgen index - have typical features of premenopausal female MS (Korhonen 2003).

The relationship between MS and PCOS is complex, as they often present together, but at the same time the association is not causative or inseparable. Although of that obesity and insulin resistance/ compensatory hyperinsulinemia are the main features of MS, but their presence is not necessary for the development of PCOS. Hence, regular menses and normal androgen levels have been observed in women with a significant degree of insulin resistance/ hyperinsulinemia (Asagami 2008). In fact, these clinical observations can be explained by the fact that women with PCOS have an intrinsic ovarian

dysfunction, which leads to an increase in the production of ovarian androgen from ovarian theca cells, in the absence of extra-ovarian factors. At the same time, without this intrinsic ovarian defect any extra-ovarian factors cannot produce overproduction of androgen by theca cells. However, hyperinsulinemia enhances inherent ovarian dysfunction. Therefore, the degree of hyperandrogenemia in susceptible women increases in association with the aggravation of insulin resistance/ hyperinsulinemia. This direct relationship between androgen production and hyperinsulinemia to some extent can describe the association between testosterone and insulin resistance, but not androstenedione levels, as it is dependent on the stability of steroidogenic defects in the ovary of PCOS. On the other hand, insulin resistance which is the main feature of MS may not prove to be present in women with PCOS - even obese ones (Kandaraki 2009). Accordingly, PCOS and MS usually coexist, but do not always overlap. The two disorders have two distinct clinical entities which appear to share some common pathogenetic mechanisms.

In PCOS, hyperandrogenemia may act as an endocrine modulator of MS. Therefore, it may act as a stimulating factor in the development of MS, not only systemically but also via local tissue androgen excess. And this make high androgens level in PCOS to have another key role beyond the one included in dysfunction of ovulation. More precisely, excess androgens may augment the negative metabolic phenotype of PCOS in a vague mode, by the exacerbation of central fat accumulation and the perpetuation of insulin resistance. In 1997, Elbers reported that long-term testosterone administration promotes the deposition of visceral fat in female to male transsexuals (Elbers, 1997). Similarly, in 1996, Lovejoy found that administration of androgen to obese postmenopausal women was associated with increases in visceral adiposity (Lovejoy 1996). While, in 2004, Gambineri reported that accumulation of visceral fat decreased with administration of antiandrogen treatment in premenopausal women with PCOS (Gambineri 2004).

Moreover, several studies have explored the impact of androgen excess in women with PCOS in the perpetuation of insulin resistance. In 1999, Cagnacci reported that insulin sensitivity in patients with PCOS improves with

suppression of ovarian androgens by administration of gonadotropin-releasing hormone agonist (Cagnacci, 1999). Similarly, in 1995, Diamanti-Kandarakis observed the same observations via antiandrogen treatment to PCOS patients (Diamanti-Kandarakis, 1995). Furthermore, in one study, androgen administration in female-to-male transsexuals has been associated with reductions in insulin sensitivity (Polderman, 1994).

The beneficial metabolic effects of Laparoscopic ovarian electrocautery in patients with PCOS has explored the potential relationship between insulin resistance, which is the most denominator of MS, and ovarian hyperandrogenism, which is the main feature of PCOS. It suggests that there is a causal interrelationship between androgens and insulin resistance in PCOS. Improvement in the insulin sensitivity has been observed in association with a reduction of ovarian androgen by laparoscopic ovarian electrocautery. This clinical finding has been supported by molecular evidence of partial reversal of insulin signalling defects in visceral fat of patients with PCOS, when treated by laparoscopic ovarian electrocautery (Seow 2007). Hence, in amenorrhoeic patients with PCOS, insulin responsiveness in adipocytes was observed to be promoted in parallel with suppression of testosterone levels after an ovulatory cycle (Marsden 1994).

Androgen may impact directly on the insulin signalling cascade, leading to a disturbance of insulin action. Attention has focused on androgens as an underlying cause for the development of metabolic disorders (Dunaif 1997). In premenopausal women, based on epidemiological studies, a positive correlation between the level of circulating androgen and insulin resistance, as well as central obesity has been observed (Seidell 1990).

In 2005, Allemand showed that addition of testosterone to cultured skeletal myotube induces insulin resistance by enhancing phosphorylation in AKT, and ribosomal S6 kinase which leads to increases in serine phosphorylation of insulin receptor substrate-1(IRS-1) (Allemand 2005). In another study, chronic treatment of human subcutaneous adipocytes with testosterone induced metabolic insulin resistance (Corbould 2007). Moreover, it is suggested that excessive release of free fatty acids (Arner 2005) in the

systemic circulation due to stimulation of subcutaneous lipolysis by androgens can induce IRS-1 serine phosphorylation (Draznin 2006).

An accumulation of fat and development of upper body obesity is due to an imbalance between the uptake and breakdown of triglycerides in abdominal adipocytes, and disturbances in lipolysis. This lipolytic system is tightly controlled by hormones, such as androgens, which could act as a putative regulatory factor (Arner 2005).

Although there is a strong link between metabolic manifestations, in the clinical, and biochemical severity of PCOS and body fat mass, yet the definite mechanisms involved are vague and not fully understood. The significant relationship between metabolic dysfunction, endocrine disturbance, obesity and the role of weight gain in the development of PCOS, justify the study of adipose tissue and the biology of its chief cells (adipocytes). Indeed, this represents an important research topic.

1.4 The link between adipose tissue and PCOS

The interest in the reproductive endocrine aspects of adipose tissue is related to the most commonly investigated and prevalent endocrinopathy in women of reproductive age - PCOS (Ehrmann 2006). In addition, about 50% of women with PCOS are obese. Indeed, obesity is responsible for unmasking the syndrome in those who might otherwise go undiagnosed (Trayhurn 2001; Barber 2006). With the increase in the prevalence of obesity, the number of PCOS cases has risen (Ogden 2006). In addition, adipose tissue is likely to play a key role in the aetiology of hyperandrogenaemia, which is the most consistent feature of PCOS. The evidence for this includes the dramatic improvement in the regularity of the menstrual cycle with reduction in weight in women with PCOS (Holte 1995).

Although the preferred treatment for some disorders associated with adipose tissue such as PCOS remains controversial, understanding the mechanisms linking adipose tissue to reproductive and metabolic health disorders is important to develop treatment approaches for helping patients (Bohler 2010).

1.5 Role of adipose tissue in the pathophysiology of PCOS

1.5.1 Physiology of adipose tissue

For the clinician and scientist, adipose tissue represents an intriguing endocrine organ, which is evidently critical for reproductive and metabolic function in women. Through both its secreting hormones, adipokines, and cytokines and via its involvement in several endocrine functions which control glucose and fat metabolism, immunity, inflammatory reaction, and reproduction (Lecke 2011) (Lecke 2013).

It is considered the largest endocrine organ in humans (Badman 2007). It is a highly specialised connective tissue, consisting of several components, involving adipocytes - the master cells of adipose tissue; pre-adipocytes, endothelial cells, macrophages, and fibroblasts (Trujillo 2000). Furthermore, it is important for survival, protects the body from trauma, and provides a major source of energy. Moreover, adipose tissue affects a number of physiological functions, including reproduction and metabolism, through an array of secreted products, such as hormones (steroid production) and adipokines (Bohler 2010).

1.5.1.1.1 Histology and differentiation

In mammals, at least two functionally different types of fat compose the adipose tissue pool: brown and white. An imbalance between energy intake and spending leads to accumulation of fat (Hossain 2007). The primary site of energy storage and of production of hormones and cytokines which control whole-body metabolism and insulin resistance is white adipose tissue (Rosen, 2006). Obesity results from excess accumulation of white adipose tissue. On the other hand, brown adipose tissue is significant for both basal and inducible energy expenditure in the form of thermogenesis; this function is mediated by the expression of the tissue-specific uncoupling protein 1 (UCP1) (Yang 2003). Brown adipose tissue has a role in whole-body metabolism and may change insulin sensitivity (Yang 2003), it also modifies susceptibility to increases in weight (Almind 2007). In rodents, it is found that brown adipose tissue is present throughout life. However, in humans it is present mainly in infants and

young children; in adults it is considered to be absent and has no other physiological relevance (Cannon 2004). However, in 1983, Rothwell suggested that in an adult human, as little as 50gm of brown adipose tissue - if it were present - could account for up to 20% of daily energy spending (Rothwell 1983). Although of that brown adipose tissue has physiological important role, but methods to assess the mass and the activity of this tissue in humans have been lacking.

Mature adipocytes are formed from pre-adipocytes after responding to various stimuli, such as glucocorticoids, the peroxisome proliferator-activated Y (PPAR-Y) agonist and insulin (Frayn 2003). This process of adipose tissue formation, known as adipogenesis, is initiated by activation of a nuclear receptor (PPAR-Y) (Rosen 2000). An increase in the size of adipocytes due to lipid droplets is known as hypertrophy, as it reaches the threshold it enhances the recruitment and differentiation of pre-adipocytes (hyperplasia) (de Ferranti 2008).

The quantity and distribution of adipose tissue is regulated by several factors, namely consumption of food, physical activity, gender, and ethnicity (Trayhurn 2005). The balance between adipose deposition (lipogenesis) and adipose breakdown (lipolysis) is reflected by the quantity of adipocytes. Moreover, 85% of the weight of adipose tissue is lipid, 95% of the content of adipocytes is triglycerides, and the remaining 5% is water, and proteins, such as adipokines-endocrine and paracrine factors, including cytokines (Trujillo 2006). As mentioned above, the most prevalent features of PCOS are insulin resistance and hyperandrogenism. The adipose tissue expandability hypothesis could explain the development of insulin resistance and obesity. In 2008, Virtue and Vidal-Puig, via adipose tissue expandability they explained the development of insulin resistance in obesity and proposed also the development of insulin resistance in fat deficiency (Virtue, 2008) (Garg 2006). This hypothesis suggests that there is a limitation in the capacity of subcutaneous adipose tissue to safely increase its mass. The ability of adipose tissue to expand in individuals is influenced by environmental and genetic factors.

When the adipocytes of subcutaneous adipose tissue begin to overfill, a lipotoxic process occurs. Lipotoxicity is defined by: elevation of free fatty acids; high levels of triglycerides; an unfavourable adipocytokines profile - which includes low circulating level of high molecular weight adiponectin and high levels of IL6 and TNF-a; and is characterised by accumulation of lipid in non-subcutaneous adipose tissue and in non- adipose organ such as liver, muscle or pancreas. Lipotoxicity has a negative impact on metabolism, particularly insulin action. Consequently, to this conception, maintenance of metabolic health is based on the ability of adipose tissue depots to accommodate caloric supply safely, without causing lipotoxicity. Therefore, the concept refers that there is a ‘‘metabolic set point’’ above this point, extra lipid storage associated with insulin resistance (Virtue 2008).

1.5.1.2 Innervation

Fat tissue is regulated by the sympathetic nervous system (Bartness 2007; Arner 2005). Activation of β-adrenergic receptors leads to stimulation of lipolysis, with generation of glycerol and free fatty acids (FFAs); while stimulation of α2A-adrenoreceptors inhibits lipolysis (Bartness 2007; Arner 2005). The sympathetic nervous system may have a role in the aetiology of PCOS (Aguado 2002; Greiner 2005; Manni 2005) (Lansdown 2012). In 2001, Heider found that the density of cathecholaminergic nerve fibres in PCOS is high (Heider 2001). In addition, there is change in the metabolism of catechol amines in adolescents with PCOS (Garcia-Rudaz, Armando et al. 1998).

1.5.1.3 Metabolism

The metabolic process of adipose tissue results either in accumulation (lipogenesis) or release (lipolysis) of lipid in the form of FFA from adipocytes (de Ferranti 2008). This process is affected by several factors including: quantity of adipose tissue (e.g. obese compared with non-obese); gender; neuronal input; the fat depot examined (e.g. intra-abdominal compared to extra-abdominal fat);

hormones including oestrogens**,** androgens, insulin, growth hormone, and cortisol; and also physical activity (Rosen 2006; Frayn 2003; Guilherme 2008; Bjorntorp 1997). During exercise when energy is needed, lipolysis is activated over lipogenesis, (Jaworski 2007). In a SAT depot, lipolytic activity is reduced compared with visceral fat, which is also more resistant to the antilipolytic action of insulin (Matsuzawa, 1994; Wajchenberg 2002; McInnes 2006).

Via the regulation of lipid and glucose metabolism, adipose tissue has an important role in glucose homoeostasis and insulin sensitivity. In men it appears that rather than obesity per se being related to the level of testosterone, adiposity – specifically the site of adipose distribution - seems to impact androgen concentrations as well as cardiovascular risk. In 2005, Kapoor documented that there is a significant inverse relationship between body fat and testosterone level (Kapoor 2005). In men with hypogonadism, which means low level of androgen, there is a reduction in the lean body mass and an increase in the fat mass, abdominal or central obesity (Seidell 1990) (Haffner 1993) (Phillips 1993) (Couillard 2000).

In 2000, Tsai found that after a 7.5-year follow-up of 110 men using scanning computed tomography (CT), there was an inverse correlation between the total testosterone levels and accumulation of visceral fat but not other fat depots (Tsai 2000). This inverse correlation between testosterone and visceral fat has been confirmed using CT and MRI (Seidell 1990) (Couillard 2000) (Garaulet 2000).

Furthermore, in 2007, Nielsen showed that the level of visceral adipose tissue was inversely associated with bioavailable testosterone in a population of young men aged 20-29 years. In this study, dual-energy X-ray absorptiometry and MRI were used to measure visceral adipose tissue (Nielsen 2007). By contrast, in 2012, Frederiksen found in aged men with low testosterone levels that after 6 months' treatment with transdermal testosterone, there was no change in visceral fat but a significant reduction in subcutaneous fat mass in both thigh and abdominal areas when evaluated by MRI (Frederiksen 2012).

However, in 2011, Hamilton observed that after a 12 months prospective observation of patients with prostate cancer treated with androgen deprivation therapy, both visceral and subcutaneous abdominal fat were increased (Hamilton 2011).

In adipose tissue, lipolysis is impacted by androgens and may be a pathway through which testosterone influences storage of fat and obesity. The major hormones regulating adipocyte lipolysis are catecholamines. Acting via activation of adenylate cyclase to produce cAMP which activates PKA, in turn stimulating hormone-sensitive lipase (HSL) to enhance the breakdown of triglycerides in lipolysis (Arner 2005). In 1980, Hansen demonstrated that in isolated fat cells from normal male rats, testosterone augmented noradrenaline stimulated lipolysis (Hansen 1980).

Moreover, in 2000, De Pergola suggested that testosterone may increase lipid breakdown by increasing the number of b-adrenergic receptors (De Pergola 2000). Data from the AR knockout mouse model shows that reduction of androgen action is associated with decreased lipolysis and is mainly responsible for the onset of obesity (Yanase 2008).

On the other hand, in 2004, Dicker found that a 50% decrease in catecholamine-stimulated lipolysis occurred from testosterone treatment of fully differentiated pre-adipocytes isolated from human subcutaneous adipose tissue (Dicker 2004). This diminishing was associated with significantly reduced expression of HSL, which is the final rate-limiting step for catecholamine-induced lipolysis. Accordingly, there may be regional variation in the action of testosterone on adipose tissue depot function (subcutaneous and visceral).

In 2002, this suggestion was supported clinically by Abate who reported that accumulation of subcutaneous adipose tissue in the truncal area is highly predictive of low plasma concentrations of free testosterone (Abate 2002). In addition, in 2007, Dhindsa found that in men with T2DM there was a significant inverse relationship between subcutaneous fat mass and free- as well as total-testosterone (Dhindsa 2007)

In addition to the effect of androgens on lipolysis it has been observed its impact on the expression of multiple key enzymes included in lipogenesis.

Dihydrotestosterone (DHT) increased the expression of hydroxymethylglutaryl coenzymes A, reductase and glycerol 3 phosphate acyl transferase (GPAT), leading to strongly induced synthesis of triglycerides, cholesterol esters and squalene (Hall 1983) (Rosignoli 2003).

In addition to androgens, oestrogen diminishes the accumulation of central fat, while increasing fat in the femoral gluteal region (Homma 2000; Mayes 2004). Moreover, cytokine tumour necrosis factor alpha (TNF-α) enhances lipolysis. In obesity, related to the elevation in TNF-α in adipocytes, the plasma FFAs are increased (Arner 2005; Moller 2000).

Moreover, adipocytes have a critical role in the homeostasis of glucose in the body. Adipocyte products may promote or inhibit glucose uptake. Leptin, adiponectin, and visfatin are adipokines which stimulate glucose uptake; whereas TNF-α, interleukin-6 (IL6), resistin, and retinol binding protein 4 (RBP4) impede glucose uptake (Rosen 2006).

The primary target tissue for insulin–stimulated glucose disposal is skeletal muscle; signals from body fat are thought to disrupt the signalling in skeletal muscle predisposing to insulin resistance (Rosen 2006; Gabir 2000). Due to the importance of adipose tissue in the homeostasis of glucose, there is a strong relationship between obesity, insulin resistance and diabetes (Bohler 2010).

Diminution in the Glucose 4 adipose-specific transporter (GLUT 4) in mice, leads to development of insulin resistance not only in adipocytes but also in the muscles and liver. Moreover, decreased expression of GLUT 4 in adipose tissue and in the skeletal muscles is associated with obesity and T2DM (Abel 2001; Kahn 1996).

Adipose tissue has a central role in metabolic health and disease, hence this tissue has become a significant target for several therapeutic approaches. A significant improvement in glucose tolerance occurs with therapy selectively directed at increasing insulin sensitivity in adipose tissue (Rosen 2006). Thiazolidinediones (TZDs) agonists for peroxisome proliferators-activated receptor (PPAR) γ are used as treatments for hyperglycaemia in type 2 diabetes mellitus (T2DM). However, due to its adverse side effects, including

fluid retention, osteoporosis, and (suggest) increased risk of cardiovascular event, its clinical used has been limited (Ahmadian 2013)

1.5.1.4 Products of adipose tissue (adipokines)

The interest in understanding the role of adipose tissue in reproduction has expanded, related to the discovery of novel adipose tissue products known as adipokines (Kershaw 2004). Adipokines are substances produced by adipose tissue. Some are produced by adipocytes, while others are formed by stromal cells, or from macrophages that migrate to fat tissue in obesity (Fain 2004).

These products include cytokines, hormones, and other factors involved in the regulation of several functions (Zhang 1994; Hu E 1996). The quantity of these products varies based on multiple factors: the metabolic state of adipose tissue (e.g. morbid obesity), adipose tissue depot (VAT compared with SAT), and cell development (pre-adipocytes compared with mature adipocytes) (Fain 2004; Halberg 2008). Some adipokines are released into the circulation, such as leptin; while others remain localised in adipose tissue, e.g. TNF-α, and produce its paracrine autocrine function (Wajchenberg 2002).

It has determined that the role of adipocytes products in several functions, such as reproduction. These products act as a link between adipose tissue and the reproductive system. For example, leptin - which is involved in the initiation of puberty by acceleration of GnRH pulsatility - also stimulates pituitary LH and FSH and facilitates the implantation process (Budak 2006). Disturbance in the concentration of these products is associated with dysregulation in the reproductive function of the body. The expression of adipokines in different compartments of adipose tissue is a selective process making fat distribution an important factor. However, the total amount of body fat is important for adipokines production (Bohler 2010).

It appears that studying adipocytes products in PCOS is an important area for investigation. This is due to the strong association of PCOS with obesity

and metabolic dysfunction, and the important role of insulin resistance in the development of PCOS, which enhance the study of these products in PCOS.

1.5.2 Adipose tissue excess (obesity)

Obesity has a significant negative impact on reproductive health. Anovulation and infertility are more frequent in women with obesity (Yannakoulia, Yiannakouris et al. 2003, Nelson, Jiang et al. 2014). Induction of ovulation by clomiphene citrate or gonadotropins may need a higher dose and longer time to induce oocyte maturation (Balen, Platteau et al. 2006). The detrimental effect of obesity on reproduction may be related to a change in hormones. McCartney, Blank et al. (2007) found that obese girls in the peri-pubertal period have an increase in total and free testosterone levels. They observed that there was a decrease in the Sex hormone binding globulin (SHBG), and an increase in insulin levels in comparison with normal weight girls of the same age CR (McCartney, Blank et al. 2007). In addition, there was also an increase in DHEAS and LH hypersecretion.

Chronic inflammation is likely a bridge between obesity and insulin resistance (Kaser, Kaser et al. 2003). Obesity is a state of chronic inflammation characterised by an invasion of adipose tissue with macrophages, and by an increase in pro-inflammatory cytokines, which are not seen in the lean individual (Pietilainen, Kannisto et al. 2006).

There is a positive relationship between an infiltration of macrophages and size of adipocytes (Pietilainen, Kannisto et al. 2006). About 30% of protein expressed in adipose tissue is related to inflammatory and macrophage-specific genes (Nien, Mazaki-Tovi et al. 2007).

An association between PCOS and low-grade inflammation has been observed, but whether it is related to PCOS per se or upper body obesity is still unclear (Rashad, El-Shal et al. , Puder, Varga et al. 2005, Repaci, Gambineri et al. 2011). The low-grade inflammatory marker, C-reactive protein (CRP), was first examined by Kelly et al. (2001). They observed that CRP was significantly high in women with PCOS in comparison with controls matched for BMI, and

inversely correlated with sensitivity to insulin. However, other studies observed that in PCOS, elevation in inflammatory markers correlated with upper body obesity and BMI rather than PCOS per se (Puder, Varga et al. 2005); Puder 2005).

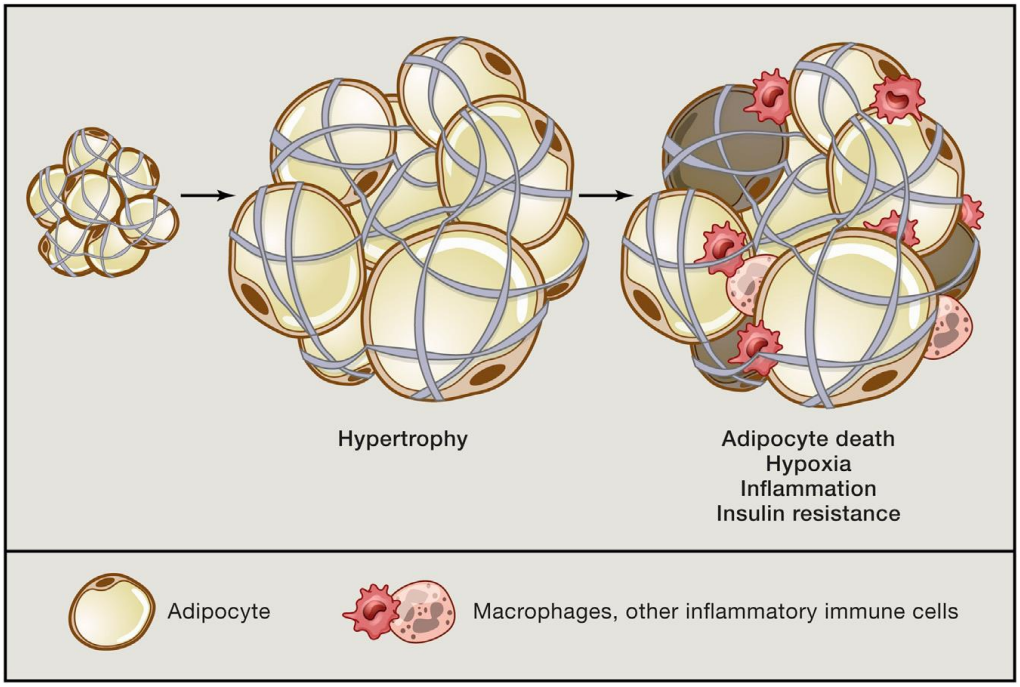


Figure 1.7 shows the interaction of adipocyte-matrix and its role in the pathology of obesity. Adipocytes produce several matrix proteins which keep the structure of the depot. Over nutrition leads to increases in size (hypertrophy), further increases in adipocytes continue until they become restricted by the matrix, which undertakes fibrotic changes. This generates changes which involve hypoxia, inflammation, and cell death, all of which cause insulin resistance. Adapted from (Budak 2006)

1.5.2.1 Adiposity and development of PCOS

Epidemiological, genetic, and pathophysiological evidence confirms the importance of obesity in the pathophysiology of PCOS in several women with this syndrome. This provides direction to research on the biology of adipocytes in PCOS.

The prevalence of obesity in women with PCOS is between 38% and 88% (Legro, Kunselman et al. 1999). It is difficult to determine the actual prevalence of obesity in PCOS for several reasons, namely the geographical and racial variability of BMI between the studies (Vialla and Partly et al. 2011); moreover, several women with PCOS remain undiagnosed (March, Moore et al. 2010). The appearance of PCOS in lean women does postulate that elements independent of obesity are critical for the development of PCOS (Barber, McCarthy et al. 2006). However, it is well defined that excess fat has a significant pathogenic role in most women with this complex disorder, and decreases in weight are associated with improvements in the outcome of the syndrome (Barber, McCarthy et al. 2006). Loss of weight by only 5% is associated with significant improvements in ovulation function, menstrual cycle, hyperandrogenic features, insulin sensitivity and metabolic function (Barber, McCarthy et al. 2006); (Kiddy, Hamiltonfairley et al. 1992). Weight loss is key in the management of obese or overweight women with PCOS, and is considered the mainstay in the treatment of obese women with this heterogeneous disorder. This evidence to supports the significant role of adipose tissue in the development of PCOS.

Although increases in weight and central obesity are common features of women with PCOS, often preceding the onset of anovulation, obesity is not one of the diagnostic criteria for PCOS (Ehrmann 2005).

A gain in weight has a critical role in the pathophysiology of PCOS in susceptible persons, where it aggravates the features of the syndrome. Obese women with PCOS often have excess androgen compared to women with normal weight (Diamanti-Kandarakis, Christakou et al. 2007). In 2005, Azziz

determined that 50% of women with PCOS are overweight or obese (Azziz 2005).

Furthermore, a common finding is visceral obesity even when the woman is not considered overweight (Douchi, Ijuin et al. 1995). It is suggested that an increase in visceral fat is an important component of the syndrome. Non-obese women with PCOS (n=30) were compared with lean women (n=30) with regular menstrual cycles and similar BMI (mean 20kg/m^2). It was found that women with PCOS had significantly more visceral fat, but a similar volume of upper body SAT (Yildirim, Sabir et al. 2003). But there are several studies showing no difference in body fat distribution (Barber 2006)

There is an obvious relationship between truncal obesity and insulin resistance (Holte, Bergh et al. 1995, Lord, Thomas et al. 2006). (Lord, Thomas et al. 2006) found that there was a significant relationship between increases in the amount of visceral adipose tissue and insulin resistance. PCOS women with IGT were more obese than women with PCOS having normal glucose tolerance (Lord, Thomas et al. 2006).

Loss of weight in women with PCOS is associated with improvements in insulin sensitivity, decreased insulin levels, reduced testosterone and increased ovulation rate (Yen, Vela et al. 1970); Guzick 1994; Huber-Buchholz et al., 1999; (Pasquali 2006). Several studies observed improvements in ovulation rate in women with PCOS with increased insulin sensitivity after administration of metformin or a thiazolidinedione (Legro, Zaino et al. 2007). Thiazolidinedione (TZDs) are synthetic ligands which can bind and activate the nuclear receptor, peroxysome proliferator-activated receptor gamma (PPARγ) (Desvergne 1999). In 2005, Stout found that in women with PCOS, administration of TZDs was associated with induction of ovulation, increasing the ovulation and pregnancy rate (Stout 2005).

In 2001, Azizz showed that in a large trial performed on 305 women, spontaneous ovulation happened over 50% of the time using 600mg troglitazone in contrast with about 10% of placebo group (Azziz 2001). In addition, in 2003, Ghazeeri found that the ovulation rate increases in PCOS patients with resistance

to antiestrogens (such as clomiphene citrate), when treated with clomiphene in association with TZDs (Ghazeeri 2003).

Hence, in women with PCOS, TZDs can improve the clomiphene citrate sensitivity, but the mechanism is unknown. It is suggested that it may have a direct or indirect action on the hypothalamo-pituitary axis in order to eliminate the negative feedback of estradiol (Ghazeeri 2003).

1.5.2.2 Distribution of obesity

Upper body obesity or central obesity indicates the accumulation of fat above the waistline and includes extra-abdominal subcutaneous adipose tissue (SAT) lying just below the skin, and intra-abdominal visceral adipose tissue (VAT) (Wajchenberg, Giannella-Neto et al. 2002). The total volume of SAT is greater than VAT (McInnes, Corbould et al. 2006). VAT consists of mesenteric, omental, and retroperitoneal fat (Wajchenberg, Giannella-Neto et al. 2002). This depot protects internal organs, such as the kidney and heart (Despres and Lemieux 2006). The products of this depot have direct access to the liver through the portal system, while SAT drains its products to the peripheral circulation (Despres and Lemieux 2006). The accumulation of intra-abdominal fat increases with age and weight gain, and decreases with exercise and weight loss (Despres and Lemieux 2006).

There are several methods to measure total body fat; one of these methods is BMI. This is a simple reliable method, especially if considered with age and gender. It refers to the ratio of weight in kg to the square of height in metres (Prentice and Jebb 2001). Based on World Health Organisation (WHO) and NIH criteria, BMI $\geq$30 defines obesity (Deurenberg and Yap 1999). However, from the biological perspective, abdominal obesity is more important than total body fat. Therefore, BMI may not be an accurate predictor of the metabolic disturbance, or reproductive dysfunction as indies that quantify fat distribution (Prentice and Jebb 2001); (Yildirim, Sabir et al. 2003); (Deurenberg and Yap 1999).

It appears that the distribution of the fat compartment may be the most important characteristic in the adverse effects of obesity (Barber and Franks 2013). In 1947, Vague was the first to make the distinction between upper and

lower body obesity morbidities (J and Vague 1947, Vague, Vague et al. 1989); (Vague 1996), and observed the change in the profiles of sex hormones and co-morbidities associated with upper body obesity. The level of androgen and oestrogen may be high in upper body obesity in comparison with lower body obesity (J 1947).

Upper body obesity is closer to an androgenic profile; thus, it is referred to as android obesity or central obesity (Pi-Sunyer 2000); (Hollmann, Runnebaum et al. 1997). Lower body obesity is referred to as gynoid or peripheral obesity as the balance of sex hormones in women is towards oestrogen (Hollmann, Runnebaum et al. 1997). Kirschner and his colleagues observed that women with upper body obesity have increased production of androgens (Kirschner, Samojlik et al. 1990).

Hyperandrogenemia and central obesity may have a bidirectional relationship. In women, a male type central obesity is encouraged by androgen (Bjorntorp 1969); (Chang, Lindheim et al. 2000). Several studies explored this relationship, in that gluteofemoral adiposity in postmenopausal women is less than upper body adiposity. This is because at this age, ovarian production of oestrogen is decreased leading to an increase in the androgen/oestrogen ratio (McInnes, Corbould et al. 2006).

Furthermore, treatment with oestrogen in postmenopausal women was associated with fat distribution similar to premenopausal women (Haarbo, Marslew et al. 1991). In women, androgen enhances central obesity and suppresses peripheral fat accumulation, whereas oestrogen has the opposite effect (Bohler, Mokshagundam et al. 2010).

On the other hand, upper body obesity may involve the elevation of serum androgen levels (Barber, McCarthy et al. 2006); (Gambineri, Pelusi et al. 2002); (Pasquali 2006). It declines with weight loss in obese women with hyperandrogenemia, and increases with increasing in central obesity (Guzick, Wing et al. 1994).

Free fatty acid (FFA) contributes to the elevation of androgen levels by contributing to insulin resistance and hyperinsulinemia, and also by increasing production of androgen precursors from the adrenal glands (Delarue and

Magnan 2007); (Hill and Melanson 1999). Infusion of short term 20% lipid in men increased the level of FFA, DHEA and androgen, before any determined affect or change in insulin resistance. The data suggest that FFA may affect the production of adrenal androgens through direct effects on the adrenal gland without a central mechanism through ACTH (Mai, Bobbert et al. 2006). Insulin also augments adrenal glands as another androgen source parallel to ovaries, by enhancing hypothalamus-hypophysis-adrenal axis (HHAA) activity at several key locations. The hippocampus is a significant mediator of HHAA negative feedback. By blocking hippocampal activity, insulin indirectly stimulates hypothalamic CRH secretion (Jacobson 1991). In addition, high insulin, compensatory hyperinsulinemia, have been associated with lower levels of SHBG, which leads to increases in the bioavailability of androgens (Wallace 2013)

1.5.3 The possible relationship between insulin resistance and adipose tissue in PCOS

In adipose tissue, adipocytes depend on insulin for intracellular glucose transport through GLUT 4. It was established that adipose tissue accounts for 10% of insulin-stimulated body glucose uptake (Smith 2002). It stimulates glucose uptake, enhances lipogenesis and suppresses lipolysis (Wilcox 2005). In addition to FFAs, adipose tissue produces cytokines which affect insulin resistance. IL6, TNF-α, leptin, plasminogen activator inhibitor, and angiotensinogen are associated with increased insulin resistance; however, adiponectin is associated with decreased insulin resistance (Devaraj 2004).

In 1930, Himsworth was the first to clearly differentiate between insulin secretion and insulin sensitivity (Himsworth 1936). He observed that a lean individual with early onset diabetes mellitus (DM) responded rapidly to insulin with decreasing blood glucose level; whereas an obese individual with late onset DM showed resistance to the lowering effects of insulin on blood glucose. In 1962, Rabinowitz and Zierler found that obese non-diabetic subjects were resistant to the impact of insulin, and had excessive levels of insulin in response to glucose infusion. From that time, it was recognised that insulin sensitivity is related to the fat content of the body (Rabinowitz and Zierler 1962). In PCOS,

they found that obesity and excess fat have an aggravating role on the syndrome's features (Dunaif 1998).

Several *in vitro* studies on adipocytes of women with PCOS have confirmed defects in post-insulin-receptor signalling. A reduction in the abundance of GLUT 4 was noted in the presence of a normal number and affinity of insulin receptors in adipocytes of women with PCOS (Rosenbaum, Haber et al. 1993); (Ciaraldi, Morales et al. 1997). Recently, it has been observed that pre-adipocytes isolated from adipose tissue of PCOS women are not intrinsically insulin resistant (Corbould and Dunaif 2007).

It was found that androgen has a role in the production of selective insulin resistance in adipocytes of women with PCOS; this action is mediated through androgen receptors (Diamanti-Kandarakis 1997). It is suggested that levels of insulin receptor substrate-1 (IRS-1) may be diminished in adipocytes from insulin resistant women with PCOS (Chu 2004; (Azziz, Carmina et al. 2006, Diamanti-Kandarakis and Papavassiliou 2006).

It is also possible that IRS-2 tyrosine phosphorylation may be decreased in adipocytes of PCOS women with insulin resistance compared with adipocytes from insulin–sensitive women with PCOS and non-PCOS (Qiu, Vadachkoria et al. 2005). A reduction in GLUT 4 in hypertrophic SC adipocytes taken from women with PCOS is another possible mechanism mediating the post insulin receptor–insulin resistance (Rosenbaum, Haber et al. 1993); (Ciaraldi, Morales et al. 1997).

The exciting data postulate a number of defective post-insulin-receptor molecular mechanisms, which may describe insulin resistance in adipocytes of women with PCOS. The definite mechanisms involved, and the determinants of adipocyte size in this disorder are still unclear. Yet, it is suggested that excess androgen associated with PCOS plays a critical role in the development of hypertrophic SC adipocytes, and the resulting insulin resistance (Villa and Pratley 2011).

1.5.4 Adipocyte steroid metabolism in PCOS

Testosterone and oestrogens are sex steroid hormones, produced mainly from the ovaries, testis, and adrenal cortex as stated above. Fifty per cent of circulating testosterone is produced from these sources, while the remainder is generated from peripheral tissues, mainly adipose tissue, which take up circulating DHEA and convert it to testosterone. This reaction is catalysed by two main steroidogenic enzymes, namely 3β- and 17β-HSD (Yildiz and Azziz 2007). Oestrogen is produced from testosterone by the action of aromatase cytochrome P450 (P450 arom) (Conley, Mapes et al. 2001).

Adipose tissue as mentioned above is considered the biggest endocrine organ in the body (Belanger, Luu-The et al. 2002). It enables synthesis, production, and inactivation of sex steroids (Bleau, Roberts et al. 1974). It was found that the concentration of these hormones in adipose tissue is higher than in the systemic circulation (Deslypere, Verdonck et al. 1985). This reflects the activity of steroidogenic enzymes in fat tissue, which consequently affects the tissue specific concentrations and serum levels of sex steroid hormones (Quinkler, Sinha et al. 2004).

In 1990, Kirschner and his colleagues observed that adiposity is associated with an increase in oestrogen production (Kirschner, Samojlik et al. 1990). The capacity of adipose tissue to produce oestrogen comes from the expression and activity of aromatase in adipose tissue. It is believed that obese women with PCOS produce high oestrogen, which could contribute to the deregulation of gonadotropin (FSH, LH) and a consequent rise in androgen production via the ovaries (Kiddy, Hamiltonfairley et al. 1992). In 2004, Quinkler considered that the production of androgen by adipose tissue may be a mechanism through which the expression of androgenicity in women with PCOS is aggravated (Quinkler, Sinha et al. 2004).

Several studies confirmed the activity of multiple steroidogenic enzymes in adipose tissue. In 2002, Corbould found a positive correlation between the ratio of type 3 17β-HSD to aromatase expression in intra-abdominal adipose tissue and the waist circumference as well as BMI. They suspected that with

central obesity, female adipose tissue is increasingly androgenic (Corbould 2002)

In 2004, Quinkler demonstrated that human female adipose tissue expresses type 5 17β-HSD (AKR1C3), a reductive 17β-HSD isozyme, which is responsible for the conversion of androstenedione to testosterone. The expression of type 5 17β-HSD is site-specific, i.e. it is higher in the SC than omentum depot. In addition, it plays a role in the differentiation of pre-adipocytes (Quinkler 2004).

Furthermore, Stewart et al. (1999) and Rodin et al. (1994) reported an elevation in the activity of 5 alpha reductase in converting testosterone to DHT in the pilosebaceous units of skin. Also, Dufort et al. (2001) characterised two enzymes from the aldoketoreductase family AKR1C, type 5 (AKR1C3) (17β-HSD), and type 3 3alph-HSD-3 (3alph-HSD-3, AKR1C2) in adipose tissue.

In addition, adipose tissue expresses several receptors which enable it to respond to afferent signals from the hormone system. In 2003, Blouin showed that the expression of androgen receptors (AR) and type 5 17β-HSD (AKR1C3) is higher in SAT, when compared with type 2 17β-HSD expression (Blouin 2003)

Masuzaki et al. (2001) and Bujalska et al. (2002) suggested that the common molecular aetiology for visceral obesity and metabolic syndrome is the increase in the expression of 11β-HSD-1 in abdominal fat tissue. The exposure of omental adipocytes to active cortisol is increased by increasing the expression of 11β-HSD-1.

1.5.4.1 Key steroidogenic enzymes in adipose tissue

Steroidogenic enzymes are those involved in the biosynthesis of sex hormone, not only in the gonads but also in the peripheral tissue, such as adipose tissue (Luu-The 2001). Numerous steroidogenic enzymes with steroid-converting activities have been determined in the fat tissue (Blouin, Richard et al. 2003).

The expression of these enzymes in adipose tissue was also proved. Locally, production of sex steroid by peripheral tissue was established through cloning multiple types of steroidogenic enzymes. These included 3β-HSD types 1 and 2 (Lachance, Luu-The et al. 1990), types 1,2,3,5, and 7 of 17β-HSD (Dufort, Rheault et al. 1999); (Labrie, Belanger et al. 1995), and types 1 and 2 of 5α-reductase. Its precursors were produced from the adrenal gland (Luu-The 2001). The expression of these enzymes in adipose tissue is potentially important in the local synthesis and inter-conversion of androgen, and the exaggeration of the symptoms and signs of PCOS with obesity (Balen 2004).

1.5.4.1.1 17Alpha-hydroxylase/17-20lyase (CYP17A1)

The synthesis of all steroid hormones follows the conversion of cholesterol to pregnenolone, which can subsequently progress down the androgen formation pathway or be converted to progesterone by 3β-HSD. CYP17 catalyses two key reactions included in the synthesis of sex steroids, which happen consecutively; (i) 17α-hydroxylase activity typically converts pregnenolone to 17α-hydroxypregnenolone and progesterone to 17α-hydroxyprogesterone, whereas (ii) $C_{17,20}$-lyase activity converts 17α-hydroxypregnenolone to DHEA (Δ^5 pathway) and 17α-hydroxyprogesterone to androstenedione (Δ^4 pathway) (Brock and Waterman 1999).

1.5.4.1.2 17β-hydroxysteroid dehydrogenase (AKR1C3)

17β-hydroxysteroid dehydrogenase (17β-HSDP) is the reductive form of 17β-HSD required to convert androstenedione to testosterone. The main active isoform in human adipose tissue is 17β-HSD type 5 (Quinkler, Sinha et al. 2004). An increase in the activity of this isozyme in the *in vitro* SAT taken from

obese women may help to explain the association of hyperandrogenaemia with obesity in women, including women with PCOS (Quinkler, Sinha et al. 2004).

1.5.4.1.3 5α-reductase

5α-reductase is expressed mainly in the skin and liver, but also in adipocytes (Thigpen, Silver et al. 1993). It converts testosterone to the more potent androgen (DHT), and is also involved in cortisol catabolism. In women with PCOS, the activity of this enzyme is enhanced, where this may offer one explanation for the higher androgenicity in PCOS. However, this cannot be explained by obesity alone (Vassiliadi, Barber et al. 2009).

1.5.4.1.4 Aromatase

The biosynthesis of oestrogen (including oestradiol, oestrone and oestriol) are synthesised from androgens through the action of cytochrome P-450 aromatase in the ovaries and the peripheral tissue (Simpson 2003); (Meinhardt and Mullis 2002). The expression of this enzyme is tissue specific, where in the ovary it is regulated by FSH and within adipose tissue by various factors, including glucocorticoids, cytokines and TNF-α (Simpson 2003). There is evidence that may support that the abnormal regulation of this enzyme may be involved in the development of PCOS. Patients with aromatase deficiency often manifest some features of PCOS such as polycystic ovaries (PCO), and elevated LH (Belgorosky, Pepe et al. 2003). In addition, common variations in CYP19 are associated with excess androgen, and with variation in the symptoms of PCOS (Petry, Ong et al. 2005).

1.5.4.1.5 11β-hydroxysteroid dehydrogenase (11β-HSD1)

It is suggested that metabolism of cortisol has a role in the development of PCOS due to an indirect action on androgen production at the adrenal gland by its feedback effect on the hypothalamus-pituitary-adrenal (HPA) axis. This is the key enzyme that converts cortisone to cortisol, and is expressed in adipose tissue (Tomlinson, Sinha et al. 2002). In obesity, the activity of 11β-HSD1 is impaired (Andrew, Phillips et al. 1998); (Stewart 1999).

In fact, it is clear that there is a complex relationship between obesity, insulin resistance, and abnormal endocrine function in PCOS. Moreover, it is obvious that adipocytes have an important effect on the metabolism of steroid hormones, particularly androgen metabolism. However, research on its role in PCOS is quite limited, and the mechanism by which it is involved in the pathophysiology of PCOS is still unclear and warrants further investigation. The activity of the steroidogenic process in adipocytes is likely to have a critical role in the pathogenesis of PCOS (Barber, McCarthy et al. 2006), particularly hyperandrogenaemia. This may be one explanation for the link between obesity and excess androgen features in women with PCOS. During the pubertal transition period in normal girls, enhanced steroidogenesis in adipocytes may be one explanation for the association seen between excess androgen and obesity (McCartney, Prendergast et al. 2006). Also, Adipose tissue is the major peripheral tissue in which androgen-metabolising enzymes are active. The endocrine and paracrine effect of the tissue may be determined by peripheral androgen metabolising enzymes. With regard to androgenic status in the presence or absence of PCOS, an increase in body weight elevates androgen levels (Pasquali 2006). There is an association between the increase in adiposity and androgenicity in both normal women and women with PCOS. This may be due to a defect in the metabolism of steroid hormones (sex steroid and corticosteroid) in adipocytes (Barber, McCarthy et al. 2006). In the last decade, there has been interest in other subjects related to adipose tissue, such as glucose and lipid metabolism, with a large number of published articles. These lie beyond the scope of this study, which seeks to investigate and understand the role of adipose tissue in the development of hyperandrogenemia and the mechanism of its development. Moreover, it is a step towards understanding the relationship between insulin resistance, androgen excess, and adipose tissue.

Acute steroidogenic regulatory protein (StAR) is one of the key link molecules in the transduction of cholesterol to the internal mitochondrial membrane, where the rate-limiting enzyme in steroidogenesis - side-chain cleavage enzyme (CYP11A1) - is anchored (Zhang 2000). Insulin seems to enhance not only StAR expression, but also CYP11A1, 17-α-

hydroxylase/17,20-lyase (CYP17A1), 3-β-hydroxysteroid dehydrogenase (3-β-HSD), and aromatase (CYP19A1) expression. This contributes to high production of androgen precursors 17-α-hydroxyprogesterone, and testosterone in polycystic ovaries in comparison to healthy ovaries (Jamnongjit 2006)

Based on previous studies (Nestler, 1998) (Apridonidze, 2005) which reported that in women with PCOS, the PI3K transduction system remains intact in the context of insulin resistance, in term of defective PI3Kase activity of INSR substrates and dysfunctional glucose metabolism, warranting ovarian androgen synthesis despite these abnormalities. In addition to that, in adipocytes, insulin seems to induce serine phosphorylation of IRS-1, which leads to inhibition of the metabolic effects of insulin associated with normal mitogenic signalling. In PCOS, it is proposed that selective insulin resistance may not only be restricted to ovarian tissue, but may also be present in adipocytes (Allemand, 2009) (Corbould, 2007). Based on these observations, in this study, the PI3Kase pathway was investigated.

Overall, the results of this research contribute further information to the literature published on reproductive physiology, especially concerning androgen metabolism, insulin action, and testosterone secretion in central PCOS adipocytes, as well as mRNA expression in both healthy and PCOS tissue.

1.9 Hypothesis:

In order to understand the role of adipose tissue in the pathogenesis and development of androgen excess in PCOS, the study hypothesises that: "Androgen metabolic pathways leading to testosterone production in subcutaneous adipose tissue are altered in women with PCOS". The excess adipose tissue androgen synthesis plays an important role in PCOS pathogenesis.

1.10 Study question:

Is there any dysregulation in subcutaneous adipose tissue sex steroidogenesis, particularly testosterone, in women with PCOS?

1.11 Aims

The overall aims of this study are to investigate androgen biosynthesis in adipose tissue in women with and without PCOS, and to test the role of insulin in this process.

1.12 Objectives:

- To analyse expression levels of the key enzymes in sex steroidogenesis, CYP17A1, (17-alpha hydroxylase/17.20-lyase (17,20α-hydroxylase)), which is responsible for providing androgen precursors in sex steroidogenesis.

- To investigate expression levels of androgen quality, determine enzyme, AKR1C3, (type 5 17β-HSD), which is responsible to convert androgen precursors (androstenedione) to testosterone, in SAT of women with and without PCOS.

- To study the expression of AKR1C3 (17β-HSD type 5) protein levels in SAT of women with and without PCOS. CYP17A1 protein did not involve in this research as was studied in adipose tissue of PCOS.

- To study the impact of insulin± LH on the expression levels of the above enzymes (CYP17A1& AKR1C3) in *in vitro* differentiated adipocytes, in women with and without PCOS.
- To investigate the insulin-signalling pathway involved in the stimulatory effect of insulin on androgen synthesis in adipocytes of PCOS. By investigating the effect of PI3-K inhibitor (LY294002) on the action of insulin on adipocytes of PCOS.
- To study the impact of insulin± LH on androgen secretion (testosterone) from *in vitro* differentiated adipocytes in women with and without PCOS.

CHAPTER 2 MATERIALS & METHODS

2.1 Ethics, subjects and samples

2.1.1 Ethics

This study was approved by the Derbyshire Ethics Committee. The Patient Information Sheet (PIS) was given to all enrolled participants, who were suitable for the study. Written informed consent from patients was given by completing the consent form after detailed discussion about the research with a clinician or researcher. All enrolled subjects gave written consent in triplicates (one each for the patient, medical record and PI records, respectively), included in the appendix. The reagents and laboratory equipment used to perform experiments of this study have been listed and included in the appendix.

2.1.2 Subjects

Subcutaneous adipose tissue biopsies were obtained from women during elective gynaecology surgery undertaken in the Department of Obstetrics & Gynaecology, Royal Derby Hospital. For the research, women were selected with PCOS who met the revised 2003 Rotterdam (ESHRE)/ (ASRM) PCOS consensus workshop group diagnostic criteria (Azziz 2005) (**see section 1.2, tab 1.1**). And from an age and BMI matched group of healthy women. All participants were of reproductive age (20-45) with a BMI of 20-35k/$m^{2.}$ Diagnosis of PCOS was based on the presence of two of the **required criteria from:**

- Chronic oligo-or anovulation
- Biochemical and/or clinical evidence of hyperandrogenism (biochemical: testosterone≥2.5 nmol/l or free androgen index≥5). We chose total testosterone at cut off point because a total testosterone is likely to be more reliable than a free testosterone known the difficulties observed with several of the assays used for a free testosterone (Rosner 2001). Testosterone ≥2.5 nmol/l, this cut off of testosterone was chosen

as testosterone values may be normal in PCOS and most testosterone values are ≤5.2nm/L (Micheal 2004).

- Ultrasound appearance of polycystic ovaries.

Exclusion criteria:

- Exclusion of other causes of androgen excess, such as late onset congenital adrenal hyperplasia (CAH). **See 1.2, table 1.2.**
- Endocrine or metabolic disease, such as thyroid and DM diseases.
- Concurrent treatment with: hormonal therapy, such as contraceptive pill or corticosteroids. Oral contraceptives will lower total testosterone, and interpretation in this case is difficult (3 months off oral contraceptives is better to obtain a 'true' testosterone value) (Micheal 2004).
- Women unable to provide written, informed consent, e.g. those unable to understand verbal or written information in English.

All women with PCOS were screened before enrolment in the research project by taking a medical history, a clinical examination, and an investigation such as pelvic ultrasound scan and a blood test. Although pelvic ultrasound may be helpful in the assessment; PCO are not specific for PCOS with more than 20% of normal women having it (Polson 1988). In the ultrasound evaluation, both the number of follicles (≥ 10 cysts measuring 2-8 mm around a dense core of stroma or scattered within an increased amount of stroma Adams 1985) and volume of ovary (>11ml) are important. Blood test includes LH/FSH ratio, a ratio ≥ 2.0 is suggestive of PCOS but is not highly sensitive or specific Micheal 2004). Testosterone levels measurement. These are considered part of routine management of women with this syndrome.

With respect to women in the control arm (non-PCOS), they had normal menstrual cycles (28-32-day cycle), normal serum levels of androgens (0.2-2.9 nmol/l) and luteinising hormone (luteal phase 1-11 nmol/l). Also, measuring of LH/FSH ratio (<2) was considered (Collins et al., 2008). The control group had no detectable cause of infertility (unexplained infertility); and did not have endometriosis, thyroid disease, neoplasm, diabetes, hypertension (BP >140/90) or renal impairment (serum creatinine >120 μmol/L).

In addition, they had not received any medication for at least three months before the study, including oral contraceptives, glucocorticoids, ovulation induction agents, antibiotics, anti-obesity drugs, anti-androgenic or antihypertensive medication. Women undergoing gynaecological surgery were all premenopausal, with an age range of 20-45 years and BMI of ≤35 $kg/m^{2.}$ Patient data is summarised in **Table 2.1.**

Parameter	Normal (non-PCOS)(n=20)	PCOS (n=15)	P value
Age (year)	32.4 (25-45)	30.5 (24-36)	Ns
BMI (Kg/m^2)	27.5 (16.7-32.2)	29.4 (20.9-31.62)	Ns
Testosterone (nmol/l)	1.5 ± 0.2	2.4± 0.5	*
Luteinising Hormone (nmol/l)	7.8 ± 1.4	29.8 ± 19.2	**

Table 2.1 Demography of all enrolled subjects. This table shows data of all subjects who participated in the study. In every section of the study, women with PCOS matched with healthy women BMI, age. Enrolled women were selected and matched. Data summarised as mean± SEM. * P<0.05, ** P<0.01.

2.1.3 Samples:

2.1.3.1 Sampling technique, collection and storage

Approximately 5 g of SAT samples were obtained from patients during elective gynaecological procedures, as mentioned above. Most of the subjects were seen at clinic and then scheduled for surgery. SC samples were extracted from fat tissue under the skin of the abdomen using a scalpel or pair of scissors during laparoscopy procedures **fig 3.1**. Reasons for surgery included one or more of the following: ovarian drilling, salpingectomy, myoma/fibroid, pelvic pain, dysmenorrhoea, and premenstrual bleeding.



Figure 2.1 shows the sampling technique. SAT samples were taken during a laparoscopy operation from fat tissue under the skin around the umbilicus using a scalpel; adapted from (Oplika 2011).

With regard to the collection of the samples after excision, the adipose tissue biopsy was directly placed in a container, moved outside the operation room or surgical suite, and divided into two parts. One part was immediately snap frozen in liquid nitrogen to protect RNA integrity. The other part was transferred into a sterile container containing Hanks' balanced salt solution (HBSS) maintained at room temperature (22-25°C) - to be used for primary adipose tissue culture.

Snap frozen specimens were then transported from the surgical suite to the university laboratory and maintained at -80°C for storage until use for molecular analyses. The time from collection until freezing was less than 20 minutes. During sample collection and transfer, precautions were taken to minimise the action of RNase with the use of RNase Away ^{TM}, wearing of gloves and mask, and the use of RNase free containers. In addition, all procedures relating to tissue preparation, isolation and culture were carried out in a class II cabinet, as samples were considered infectious material.

All buffers, composition, and reagent used during this project including suppliers and their addresses are listed in the appendices.

2.2 Gene expression analysis assay

The following demonstration shows the steps of studying the level of mRNA expression, from first step until data analysis:

- Subjects
- Sample collection and storage
- Preparation for isolation or extraction of total RNA from adipose tissue and from adipocytes
- RNA extraction procedures
- Assessment of the quantity and quality of extracted RNA
- Evaluation of the integrity of isolated RNA
- Preparation of c DNA template (Reverse transcription reaction)
- qRT-PCR assay development
- Validation of references genes for normalisation

- Experimentation
- Gene expression data analysis

2.2.1 RNA extraction by Trizol reagent method

Gene activity is controlled at transcriptional and post-transcriptional levels; therefore, the most important method to assess such activity is by determining the level of the corresponding mRNA. Thus, RNA needs to be isolated first, and then the mRNA of the gene of interest analysed using several techniques: Northern blotting, fluorescent in situ hybridization (FISH) and DNA microarray as well as real-time Reverse Transcription–Polymerase Chain Reaction (RT-PCR) (Altman 2006). Successful and reliable use of the methods of molecular biology, such as RT-PCR, needs a sufficient amount of high-quality RNA taken from adipose tissue (Lacinova, Dolinkova et al. 2008).

There are several methods to isolate RNA from tissue. The phenol-based extraction method (ambion.com) is one of these procedures which utilises Trizol reagent, and was used in this research.

2.2.1.1 RNA isolation from frozen subcutaneous adipose tissue (SAT) samples

Frozen SAT samples, which had been already collected and stored at -80 °C, where processed to extract total RNA and then perform gene analysis. This method includes the following steps: homogenisation, phase separation, and then RNA precipitation, washing and dilution.

2.2.1.1.1 Homogenisation

This was performed by weighing and transferring 120mg to 130mg from the frozen adipose tissue sample to a Falcon tube (14ml), to which 1ml of Trizol reagent was added for each 120-130mg of tissue. The sample was homogenised by a Power homogeniser (Janke & Kunkel, IKA Labortechnik), because mRNA is thermal instability, the homogenization step was performed on ice. The homogenate was transferred to an Eppendorf microcentrifuge tube and

incubated at room temperature (15-30°C) for 5 minutes to allow and ensure complete dissociation of nucleoprotein complex (**fig 3.2**).

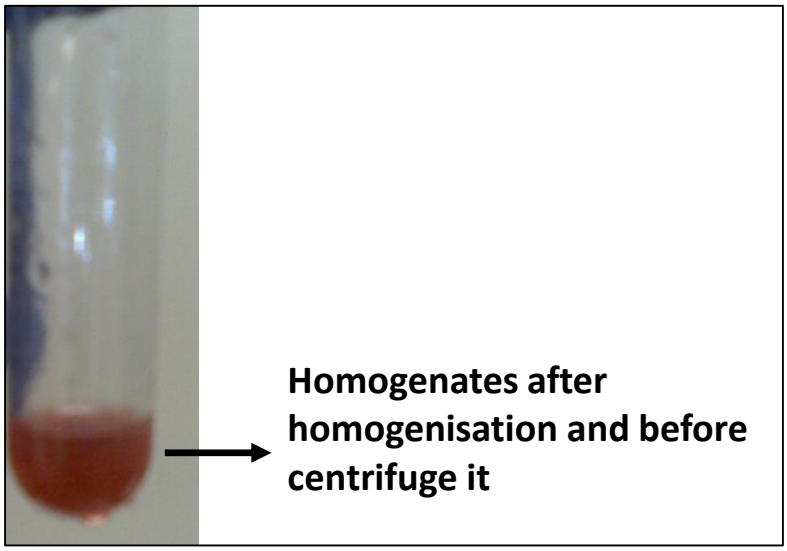


Figure 2.2 shows homogenates of a frozen adipose tissue sample, after addition of 1ml of Trizol reagent and homogenisation using a power homogeniser.

2.2.1.1.2 Lipid removal

Fat tissue contains high lipids, which interfere with precipitation of RNA and result in a contaminated pellet. In order to remove insoluble materials, such as fatty material - samples were centrifuged at 12,000 g for 15 minutes. The resulting top surface layer was fat, which was removed. The second lower layer proceeded to the next step (**fig 3.3**).

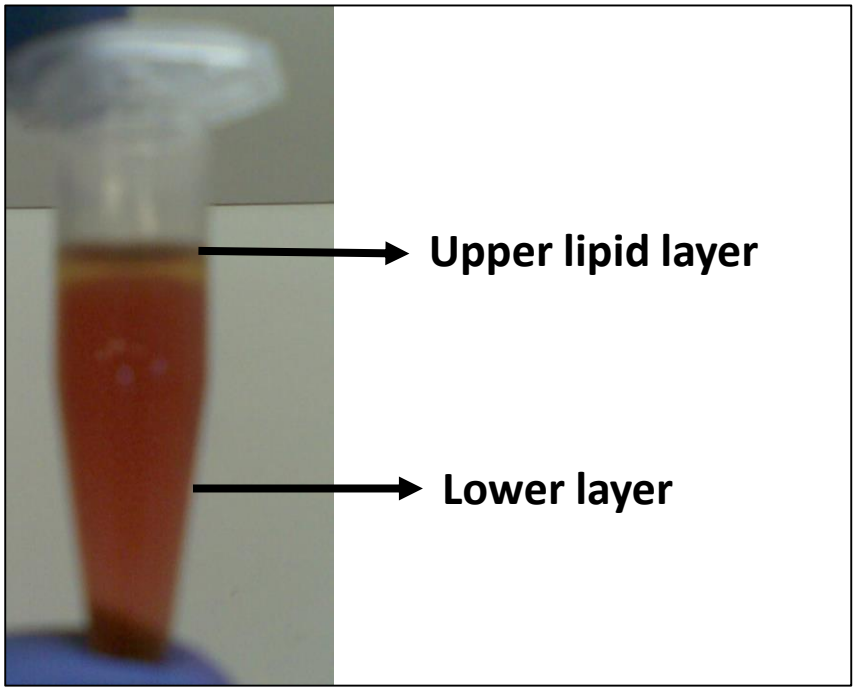


Figure 2.3 Lipid removal Step. Fat content of the tissue was removed from the sample by centrifuging the sample before the phase separation stage, in order to remove lipid, which interferes with RNA precipitation. Two layers are seen after centrifugation; the upper lipid layer (oil-like) was removed, while the lower layer proceeded to the next step of RNA solation (phase separation stage).

2.2.1.1.3 Phase separation

For phase separation, 0.2 ml of chloroform was added (for every 1ml Trizol), to the remaining lower layer after removing the upper lipid layer. Then the sample was mixed well by shaking thoroughly by hand for 15 seconds. During this period, mixing with vortex was avoided to diminish the risk of contamination with DNA.

After mixing, the sample was incubated at room temperature for 3 minutes and centrifuged for 15 minutes at centrifugation speed 12.000×g at 4°C; this is essential for phase separation. After centrifugation, three layers were seen: a colourless upper aqueous layer (RNA) consisting of 60% Trizol volume, an interphase (DNA), and a lower red phenol chloroform layer (**fig 3.4**). The upper aqueous layer (approximately 540µl) was carefully transferred (without disturbing the interphase layer) to a new RNAse-free 1.5 ml Eppendorf microcentrifuge tube for the next step, namely precipitation of RNA.

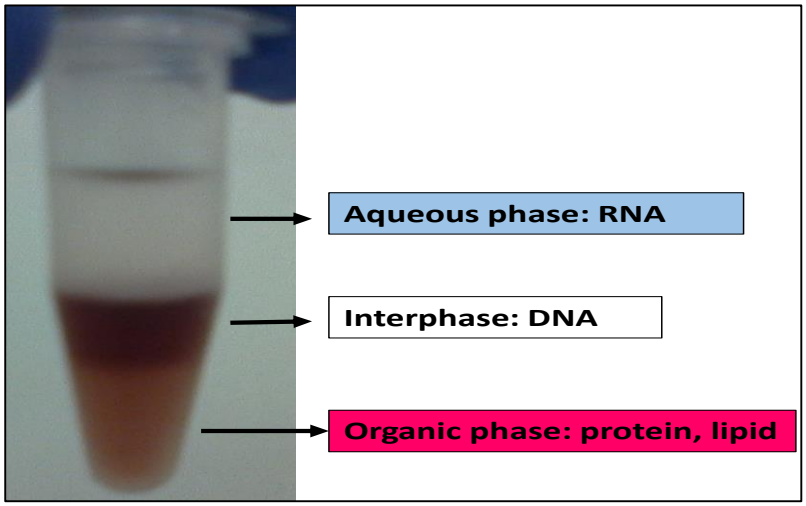


Figure 2.4 Diagram shows the separation of the mixture after centrifugation into three layers. The top aqueous clear layer where the RNA is suspended, the volume of this layer is 60% of volume of Trizol; middle interphase layer where DNA is found; and lower red organic phase where proteins and lipid are present.

2.2.1.1.4 Precipitation of RNA

RNA in the aqueous phase was precipitated by adding 0.5ml of isopropyl alcohol (for 1ml of Trizol reagent). After that, the sample was incubated at RT for 10 min, and then centrifuged at no more than 12.000×g for 10 min at 4 °C. RNA was invisible before centrifugation, but after a very small gel-like pellet was seen on the side and bottom of the tube.

2.2.1.1.5 Washing of precipitating RNA

Prior to washing, the supernatant was removed carefully and completely. The pellet was washed once with 75% ethanol, and then the sample was mixed by vortex to spread the washing solution on the membrane of the tube to increase RNA yield; and then centrifuged at 7.500×g for 5 min at 4 °C. The supernatant was carefully removed, and the RNA pellet was left to dry with air for 5 min. Over-drying was avoided to prevent any difficulty in dissolving the RNA pellet.

2.2.1.1.6 Dissolving isolated RNA

Isolated RNA was dissolved by adding 100µl RNAase-free water. Finally, a preparation of RNA free of DNA and protein was obtained.

2.2.2 RNA isolation from monolayer cell culture

After preparation of primary cell culture, by isolation the pre-adipocytes from fresh adipose tissue samples were maintained and *in vitro* differentiated. The differentiated cells (mature adipocytes) were treated and prepared for gene expression analysis. Then, the total RNA was isolated from these cell culture monolayers in the 24 well plates using the same technique (mentioned above). However, here the volume of Trizol reagent added was based on the surface area of the plate, not on the volume of the tissue sample, generally for every 10cm^2 of surface, 1ml of Trizol was added.

In addition, the other difference between the isolation of RNA from frozen adipose tissue samples and from monolayer cell culture, is that here there

was no need to centrifuge the homogenate before phase separation, because of insufficient lipid to interfere with RNA precipitation. Instead, the homogenate was taken directly to the phase separation stage. The other steps (phase separation, RNA precipitation, and RNA washing) are the same as described above.

2.3 RNA quality assessment

2.3.1 Quantity, and purity using the NanoDrop Spectrophotometer

The concentrations and purity of extracted total RNA were measured by spectral photometry using a NanoDrop-1000 spectrophotometer (Labtech). It was qualified by determining the ratio of absorbance at wavelengths, 260 and 280 nanometre (nm). The aim from assessing the quality - in terms of quantity and purity of the RNA - is to confirm the sample integrity for downstream gene expression studies, such as RT-PCR analysis.

NanoDrop ND-1000 spectrophotometry gives accurate and reproducible measurements within the 220-750 nm spectral range. In order to measure the concentration and purity, it requires only 1 µl of RNA solution, which is one of the greatest advantages of this method. Furthermore, it is simple and commonly used. On the other hand, it is a considerably less sensitive technique than other methods that can be used to assess isolated RNA, such as Fluorescent day (Ribo Green) and Agilent 2100 bio-analyser. Moreover, it cannot assess the integrity of extracted RNA when compared with the Agilent 2100 bio-analyser, which can quantify and assess RNA integrity at the same time - but is not available everywhere (Applied Bio System 2006).

For assessment, Launch NanoDrop software was used. The application was started after equipment preparation, and became ready to measure RNA quantity and quality.

RNA concentration in the sample was measured by application of 1 µl of total RNA sample to the pedestal and clicking on measure with the result appearing on the computer.

Generally, RNA quality was determined by measuring the absorbance at 260nm (A260), and 280nm (A280). Isolated RNA was considered pure if the 260/280 ratio range was 1.8-2.0. The presence of co-purified contaminants leads to a low ratio, with greater absorbance at 280 nm or near to 280nm such as protein and phenol. With regard to the concentration, a good yield is achieved if the result is >300ng/µl (Invitrogen).

2.3.2 RNA integrity using denaturing 1% agarose gel electrophoresis

The integrity of RNA was examined by separating isolated RNA on denaturing agarose gel electrophoresis containing Ethidium Bromide (EtBr)(Afcs solution protocol ID ps00000076) (**fig 2.5**), which binds to the RNA and is then detected under ultraviolet light (**fig 2.6**).

In intact isolated RNA, two distinct and sharp bands were visualised, representing 18S r RNA small (2 kb) and 28S r large (5 kb) ribosomal RNA. Extracted RNA after assessment was stored at -80 °C to be used for the next assay (RT-PCR).

The procedures of this experiment were as follows:

Agarose gel preparation: Agarose gel 1 % was prepared by dissolving 1g of agarose powder in 100ml of TBE (Tris/Borate/EDTA) buffer (1%), and heating in the microwave for 2 minutes on high power to ensure dissolving of gel. 1µl of 25 mM EtBr was added to the cooled 1% agarose solution and mixed well. Toothcomb (1.5-mm slots) was placed into the tray of the horizontal minigel electrophoresis system. Then 80ml of agarose solution was placed into the tray until it covered the top of the wells. The agarose was allowed to solidify by leaving it for at least 45 min at room temperature; the tray was placed with gel into the electrophoresis minigel box.

Buffer preparation: TBE buffer was prepared by dissolving TBE (these are measurements to prepare 500ml) 54g Triz, 27.5 g Boric acid, and 3.5g EDTA (20 ml) in about 500ml of distilled water and mixed for 5-10minutes. pH was checked and adjusted to 8 (to be alkaline). The prepared buffer was diluted 10 times by adding 100ml of prepared TBE buffer to 900ml of distilled water to produce 1000ml (1 litre) X10.

Sample preparation: RNA samples for assessment were prepared by mixing 5µl of total RNA with 2µl of staining dye to a total volume of 7 µl. After removing the comb from the solidified gel, RNA samples were loaded, 1kb plus DNA ladder (kb ladder) was also applied to the first well; other samples were placed into the subsequent wells. The electrodes were engaged to the minigel box, and then the gel was run at 90 V for 45 minutes.

The images of RNA in the gel were visualised with a UV light box and recorded using an Olympus camera.

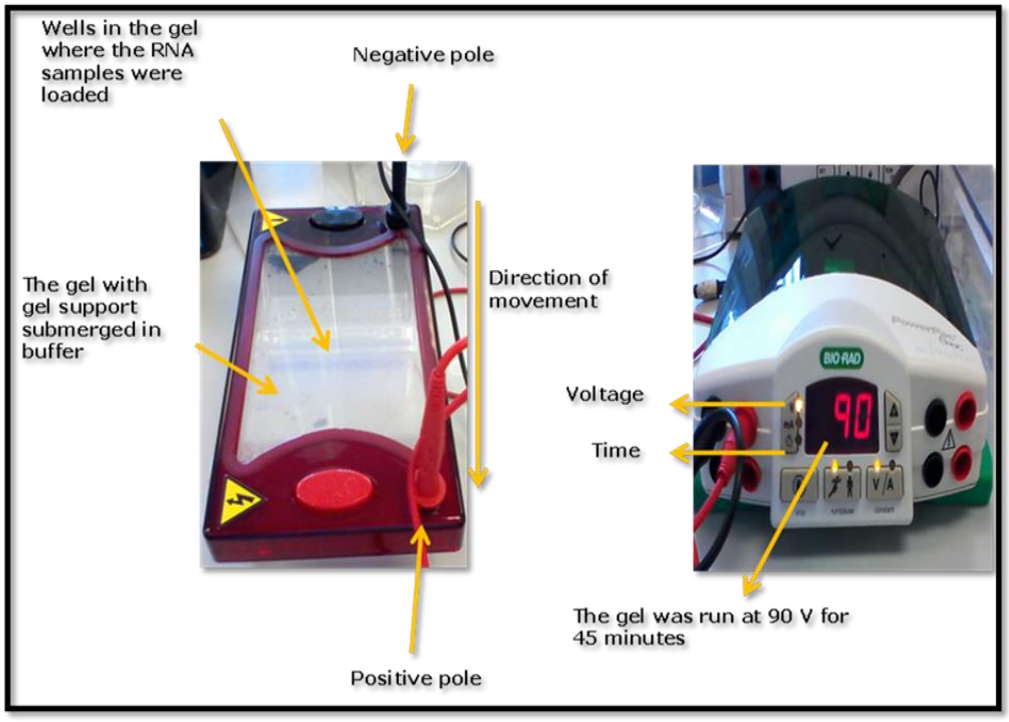


Figure 2.5 Agarose gel electrophoresis was used to assess the integrity of extracted total RNA by the Trizol reagent method from SAT of women with and without PCOS. Total RNA isolated using the Trizol procedure was loaded by pipette on agarose gel wells in a horizontal gel apparatus (left picture), which consisted of a gel slab submerged in buffer solution. The gel was run at 90 V for 45 minutes (right picture). RNA (negatively charged nucleic acid) will move toward the positive electrode, and the separation of RNA molecules is based on size - so larger molecules will move more slowly than smaller molecules.

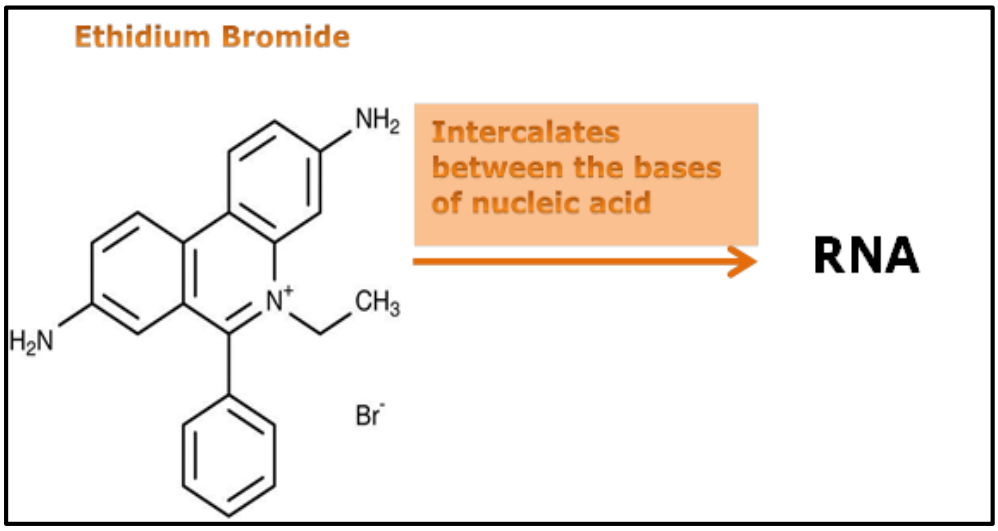


Figure 2.6 Ethidium Bromide (EtBr) molecules intercalate between the bases of RNA. Binding of EtBr to the RNA will lead to fluorescence of the gel when it is exposed to or illuminated with UV light.

2.4 Reverse transcriptase reaction: Synthesis of c DNA template

Reverse transcriptase reaction is a reaction in which RNA is reverse transcribed into c DNA. The c DNA is used as a template for quantitative PCR amplification (qRT- PCR), which is a technique used to investigate gene expression. The accuracy in the analysis of gene expression needs good reproducibility of reverse transcriptase reactions.

The sensitivity, dynamic range and specificity of the reverse transcriptase are key parameters which determine the robustness of the reverse transcription process.

An applied Bio-system kit was used to make c DNA in a total reaction of 20µl. The procedures have been described in detail below. In addition, to synthesise c DNA, no reverse transcription control, -RT (negative controls), and no templet control, NTC (just nuclease free water), were setup. This is a very important step in the synthesis process, as it provides a mechanism to control for external contamination, genomic DNA contamination or other factors which can result in a non-specific increase in the fluorescence signal., and was prepared with the same mix, which is used for the investigated group.

2.4.1 Materials

- **RNA template**.

Successful c DNA synthesis is based on the integrity and purity of the RNA template. Therefore, as described previously, during RNA preparation it should be free of any DNA and RNAase contamination.

For c DNA synthesis, the maximum amount of template RNA is 1µg. This includes all RNA present in the sample; for example, r RNA, carrier RNA and other possible RNA in addition to the target RNA.

- **Reverse transcriptase**.

This is an enzyme providing good sensitivity in qRT-PCR. The most commonly-used enzymes are Moloney murine leukaemia virus (MMLV) and

Avian myeloblastoma virus (AMV). MMLV and its derivatives are used when long or full-length c DNA transcript is required.

- **RNase inhibitor**.

This is involved in the mix with reverse transcriptase to prevent any contaminating RNase present in the RNA sample from destroying the RNA.

- **RT primers**.

The quantification of certain target genes may be affected by the choice of primers. **Random hexamers** and **oligo (d NT)** are **primers** used for c DNA synthesis.

Random primer is considered a good starting point in c DNA synthesis as it enables transcription of total RNA (mRNA, r RNA, t RNA and *in vitro* transcribed RNA) producing c DNA, which includes the whole transcript. The amount of random primer per 20µl RT reaction can be optimised between 200ng-400ng. In addition to random primer, **Oligo (dN T)** was also used to transcribe poly $(A)^+$ RNAs, the recommended amount of this primer per 20µl RT reaction is 100ng, which can be increased to 1µg.

- **Minus RT control (-RT)**.

The qRT-PCR experiment needs –RT to test for DNA contamination, such as genomic DNA or PCR products from a previous run. For this control, the reaction contains all reaction components for RT, except reverse transcriptase. Thus, a RT reaction should not occur in this control, i.e. if PCR amplification is seen, it comes from DNA contamination.

2.4.2 Methods

120ng of total RNA was reverse transcribed; each reaction was carried out in 40µl final volume. The reaction mix consisted of RT buffer, dNTP mix (0.8µl), random primer (2.0µl), RNase inhibitor (1.0µl), nuclease free water (3.2µl), and Reverse Transcriptase (1.0µl). The reverse transcriptase reaction on

the PCR machine was undertaken at 25°C for 10 minutes, 37 °C for 2 hours, and 85°C for 5 minutes. Each reaction was performed in duplicate: one containing the RT enzyme (+RT) and the other without (-RT), in which reverse transcriptase (RTase) was substituted with water.

2.5 Determination of assay efficiencies

After optimisation of RNA extraction procedures, obtaining RNA suitable for the qRT- PCR technique, and c DNA synthesis; the efficiency of the gene assays for all relevant genes, (for gene assays **see table 3.2**, (3 reference genes and 2 target genes) was determined by the creation of standard curves. The efficiency and optimisation of the qRT-PCR reaction was evaluated by generating standard curves of three selected reference genes. The standard curves were generated using a 10-fold dilution of templates amplified on the real time system (a Chromo 4^{TM} System controlled by Opticon Monitor software). Each reaction contained 2μL cDNA solution, 10μL 2x Taqman Universal Master Mix, 0.7μL Taqman Gene Expression Assay and 7.3μL H_2O. Each reaction was performed with initial denaturation at 95°C for 10min, followed by 50 cycles of denaturation at 95°C for 15s, and annealing/extension at 60°C for 1min. Each dilution was assayed in triplicate. For determination of assay efficiency, a standard curve was created in Excel (Microsoft, Redmond, USA) with the Ct plotted against the log of the starting concentration of the template (c DNA) for each dilution.

Then a trend line was created using linear regression, the assay efficiency in % was calculated using the equation $E\%=(10^{(-1/slope)}-1)*100$ (Pfaffl, Tichopad et al. 2004).

The hallmarks of an optimised qRT-PCR assay are a linear standard curve (R2 >0.980), high amplification efficiency (90-105%), and consistency across replicate reactions. The most common cause of reaction >105% is pipetting error during serial dilutions.

Genes	Gene name	Assay ID
	ACTB	Hs 01060665_g1
Reference genes	GAPDH	Hs 02758991_g1
	LPR10	Hs01047362_m1
Target genes	CYP17A1	Hs 01124136_m1
	AKR1C3	Hs 00366267_m1

Table 2.2 Assay ID for reference and target genes of this study. Reference β-Actin (ACTB), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and receptor-related protein (LPR10); and target 17, 20 α-hydroxylase (CYP17A1) and 17-β hydroxysteroid dehydrogenase (AKR1C3) genes. Adapted from (ThermoScientific).

2.6 Reference gene detection

For determination of references genes in SAT derived from non-PCOS (n=5) and PCOS women (n=5) matched for age and BMI; based on literature studies - six reference gene candidates with certain gene function were selected and examined for the stability of their expression (de Jonge, Fehrmann et al. 2007); **see table 2.2** for gene analysis assay ID. After RNA extraction and c DNA synthesis, q PCR of each sample was performed in triplicate for each of these following selected genes: β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH**),** receptor-related protein (LPR10), hypoxanthine phosphoribosyl transferase 1 (HPRT1), beta -2-microglobulin (B2M), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHAZ).. The q PCR reaction consisted of 2 µL 2x Taqman Universal Master Mix, 0.7 µL Taqman Gene Expression Assay and 7.3µL H_2O. The reaction was run on a Chromo $_{4TM}$ System (Biorad) (**Table 2.2**).

Subsequently, with the help of Genex software (Multi d analyses software, Göteborg, Sweden) reference genes were determined using the Genorm and Normfinder algorithms guided by the Genex manual. First, the raw data was pre-processed by identifying and excluding outliers using Grubbs test; then the average of technical replicates was calculated. Data was analysed by Normfinder considering the group of samples (PCOS and non-PCOS). The intergroup variation was determined by examining the gene that showed significantly higher variation than the other genes, affecting the general mean. After exclusion of these genes, the analysis was repeated until all genes showed the same variation. Using Genorm, data containing the reference gene candidates were analysed.

2.7 qRT- PCR of target genes (CYP17A1 and AKR1C3)

Quantitative real time polymerase chain reaction (qRT-PCR) was carried out to analyse mRNA expression of two steroidogenic enzymes (CYP 17A1 and AKR1C3). The reaction was performed using 2µl c DNA template which was made from total RNA of 16 SAT, (n= 8) from women with PCOS and from an age and BMI matched group of healthy women (n=8), selected from all

participate of study (**see table 2.1)**. Each reaction was usually performed in triplicate, including both +RT and –RT samples and a no template control (NCT) in which template c DNA is replaced with distilled water, to check for any contamination in the PCR components.

qRT-PCR was performed on Taq-Man chemistry, and as previously described (Catalán et al. 2008) on a 7900 Real–Time PCR system (Applied Bio system) in a total volume 20µl in 96-well plates containing TagMan Universal PCR Master Mix buffer (Applied Biosystems).

All results were normalised with selected references genes GAPDH, ACTB and LRP10. Amplification was carried out in a thermal cycler under the following conditions: 95°C for 10min, then 40 cycles of denaturing at 95°C for 15 seconds, followed by annealing/extension at 60°C for 1 min.

2.8 Analysis of qRT-PCR data

Data were expressed as Ct values (= cycle number at which the logarithmic PCR plot crossed a calculated threshold line) (**fig 2.6**), and were used to determine delta Ct values (ΔCt) (=the difference between the two Ct values of PCR for the same initial template amount (Vandesompele et al. 2009). This was calculated using the **comparative threshold** (Ct) method to compare **the relative amount** of the target sequence to the values of chosen reference genes. The result was given relative to the reference value (Vandesompele et al. 2002), and was calculated by GenExe software.

The key advantages of using the relative comparative Ct method are: (i) elimination of the need for a standard curve, which means more wells for the samples are available; and (ii) removing or avoiding the negative effects of any dilution mistake made in creating the standard curve samples. However, before using the delta method for quantitation, validation of the experiment was performed to demonstrate the efficiency of the performance of the reaction. This was done by processing serial dilutions of the chosen reference genes in a PCR reaction, and then the efficiency of the reaction was assessed (Applied Bio system guide).

Standard curve or absolute quantification is an alternative approach for analysing quantitative data. This method is used when it is important for the design of experiment and where the aim from of the project is to measure the level of template in a sample - such as measuring the viral load in a sample, using cloned genes etc. To create standard template, types of sources can be used such as, genomic DNA, and c DNA. The most significant consideration in this approach of absolute quantification, is that the primer set is validated to work efficiently with standards and the experimental source material. The standard curve is created by plotting the log of the initial template copy number against the Ct obtained for each dilution, after amplification of the standard dilution series. The quantity of initial copy number can be obtained by comparing the Ct values of unknown samples to the standard curve (Strata gene guide)

Figure 2.6 amplification curve of examining samples explains how real time PCR works. In this curve, on the X-axis the PCR cycle number is shown. On the Y-axis the fluorescence from the amplification reaction - which is proportional to the amount of amplified product in the tube is shown. From the curve, two phases are observed, an exponential phase followed by a non-exponential plateau phase. The amount of PCR product approximately doubles in each cycle during the exponential phase. However, the components of the reaction are consumed with progression in the reaction, and eventually one or more of the components become restricted. At this stage the reaction becomes slow and approaches the plateau phase. Firstly, fluorescence persists at background levels, and rises in fluorescence are not determined (cycle 1-22 in fig 2.6). Enough accumulation of amplification products is associated with a detectable fluorescent signal. The cycle number at which this happens is called the threshold cycle, or Ct. As the Ct value is measured in the exponential phase when reagents are available, real-time q PCR can be used to reliably and accurately compute the initial amount of template found in the reaction.

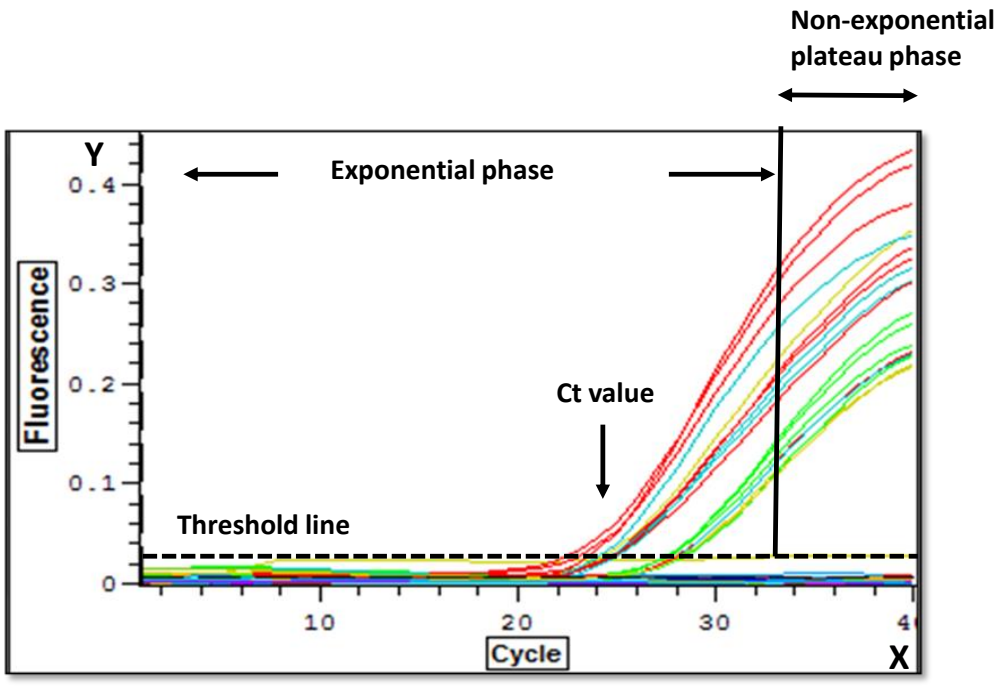


Figure 2.6 Amplification curve for the isolated adipose tissue RNA gene expression analysis using quantitative Real-time PCR (qRT-PCR). 120ng of the isolated RNA was reverse transcribed using Reverse Transcriptase (Applied Bio system) with random primers as described in the text. Gene expression of CYP17A1 and AKR1C3 was analysed via qRT-PCR performed with TaqMan chemistry using the 7900 Real-time PCR system and universal cycling conditions. The CYP17A1 and AKR1C3 TaqMan primer-probe pairs were obtained from Applied Bio systems.

2.9 Investigating AKR1C3 protein expression by Western blot (WB)

Western blot (WB), or immune blot, is a widely used semi-quantitative technique to detect a specific protein from a given sample. It uses gel electrophoresis to separate already denatured proteins based on their size/weight. After protein separation, they are transferred to a nitrocellulose membrane and probed with specific antibodies to study target protein (antigen, antibodies reaction). The following sections describe the technique in detail.

In this study, protein expression of 17 β-HSD type 5 (AKR1C3) was compared in women with and without PCOS. Lysate from placental tissue was used as a positive control, as it is known that placenta expresses 17 β-HSD type 5 protein. The presence of a positive control in the procedure helps to understand what worked and what may have happened wrong. A positive result from the positive control even if the samples are negative indicates the technique is optimized and working, and it will clarify that any negative results are valid (Liu 2013).

Before starting WB, it is very important to prepare the sample lysate. For lysate preparation there are different protocols each according to the tissue type.

2.9.1 Frozen adipose tissue sample homogenisation.

SAT samples stored in the freezer at -80°C (**see section 2.1.2**) were processed for protein extraction within the Clinical Sciences Lab.

Frozen samples (500mg) were chopped in 4ml of homogenisation buffer, consisting of sucrose 300m M, tris base 25m M, pH 7 (Invitrogen Life Technologies); monothioglycerol 10m M, EDTA 1m M protease inhibitor cocktail 0.5% (V/V), 2.5% (V/V) Igepal CA360 on ice; and homogenised using a power homogeniser (Hiedolph Diax 900) within a fume cabinet until the sample was homogenised completely.

Homogenate was then centrifuged at 12.000×g for 30 minutes at 4°C. The aim from this centrifugation was to remove lipid, which was seen on the top of the supernatant. After removing the lipid layer, the homogenate was

centrifuged again at 12.000×g for 30 minutes at 4°C, supernatant was removed and collected in an Eppendorf tube and stored at -80°C until required.

2.9.2 Estimation of protein concentration using the Bicinchronic acid assay (BCA)

It is important to measure the exact concentration of proteins in a solution in order to load equal amounts of protein into the wells. There are several methods to determine protein concentration. All methods have advantages and disadvantages, and it is important to choose by considering the following aspects: specificity, sensitivity, the measurable range of concentration, the accuracy, the nature of protein to be measured and the time needed for the measurement.

The Bradford protein assay is a widely used protein assay dependent on the ability of the Coomassie Brilliant Blue dye to bind to proteins in acidic solution (through electrostatic and van der Waals bond) leading to a shift of the absorption maximum of the dye from 465 to 595 nm. The advantages of this method are that it is a highly sensitive fast method to measure 1-20µg of protein. However, its work based strongly on the composition of amino acids and which it stains the cuvettes used.

The other means of protein concentration measurement is Bicinchronic acid method (BCA), which is one of the most common method used. The advantages of this method are it needs only few materials (Tris, and amino acid buffer) interfere with it, it can perform within a short time, and is not based on the amino acid composition of proteins. On the other hand, its sensitivity is low and it needs at least 1mg of protein (Thermoscientific.com). This method was used in this study.

Measurement of protein concentration using the BCA method is based on the reduction of Cu^{2+} to Cu^{+} in the presence of protein, producing a colour change quantified by measuring absorbance at 562nm by spectrophotometry.

In order to estimate protein, first, protein standard is prepared and a comparison can be made so protein can be estimated. Protein standard was prepared by serial dilution of a 10mg/ml stock solution of Bovine serum albumin

(BSA) (0, 9% NaCl + 10µl of BSA 10mg/ml) to produce BSA concentrations of 0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1mg/ml.

The standard protein samples were loaded in duplicate along with unknown samples in a 96 wells plate; 200µl of BCA working reagent (4% copper sulphate ($CuSO_4$) in BCA reagent) was added to each well and incubated at 32°C with gentle agitation for about 10 minutes.

Using spectrophotometry, the absorbance of the standard and unknown samples were read at 562nm on a plate reader (Thermo Electron Corporation Multi scan Spectrum).

The standard curve was constructed using the results of spectrophotometry (**fig 2.7**). By application of this protocol, the results were collected and examined against linear regression to estimate protein concentration.

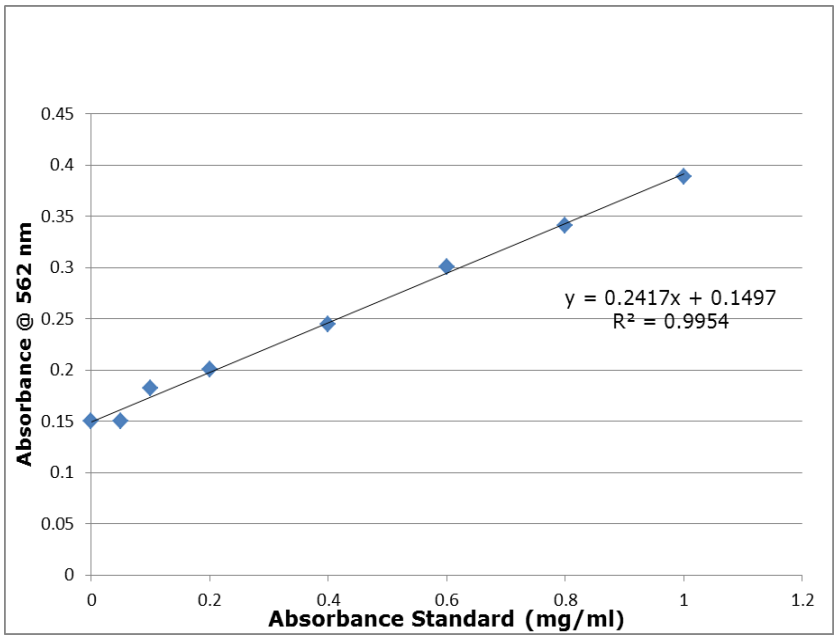


Figure 2.7 shows the absorbance of known concentrations of protein. These were used to construct a standard curve, then used to calculate the unknown concentration of protein in the sample.

2.9.3 Gel preparation (SDS-PAGE)

For preparation of gels for WB, glass gel plates were used. First, these were cleaned using 70% ethanol and then secured (two plates) using petroleum jelly within the cast clamps. The percentage of the acrylamide was chosen based on the molecular weight of the protein being separated (7, 10, 12%). For protein with low molecular weight (10- 15KDa), a high percentage of acrylamide was used, while a low percentage of acrylamide was used for proteins with a high molecular weight.

The resolving gel was prepared using 12% acrylamide stock (30%); resolving gel buffer, consisting of d H_2O, Tris 1.5M PH 8.8, 0.1% (v/v) SDS; 0.001 % (W/V) ammonium persulphate, and 0.1 % (V/V) Temed (appendix). After preparation, this was syringed between two plates and left to set for about 30 minutes; a fine layer of butanol was added on top of the resolving gel to prevent oxygen exposure and promote polymerisation of the resolving gel.

The stacking gel was prepared using 4% acrylamide stock (30%); stacking gel buffer consisting of d H_2O, Tris 1M PH 6.8, 0.1 % (V/V) SDS; 0.001% (W/V) ammonium persulphate and 0.1 % (V/V) Temed. After washing off butanol using d H_2O, stacking gel was immediately added followed by insertion of a lane comb - and left to set for about 30 minutes. For this, the Bio-Rad mini- protein II electrophoresis units were used.

2.9.4 Samples preparation and loading

The protein concentration was adjusted to 20mg/ml using Laemmli buffer. The investigated samples were denatured by exposing them to a high temperature (a hot plate set) of 95°C for 5 minutes. 100 µg of protein was added to each lane. In the first lane, 10µl of Kaleidoscope pre-stained molecular weight marker was added to facilitate the detection of the size of proteins.

2.9.5 Electrophoresis (separation)

After loading of proteins, the gel was placed into running buffer consisting of Tris (25m M), glycine (192m M), and d H_2O. This was run for 1 hour at 40 mA (constant).

2.9.6 Electro-transfer (blot) step

After separation, proteins were electro-blotted onto nitrocellulose membranes on ice cold transfer buffer which included Tris base (24m M), glycine (80m M), and 20%(V/V) methanol. Within the BioRad miniprotein blotting system, a sandwich of gel and nitrocellulose was added, and a charge of 100V run for about 2 hours at 4 °C. The transfer process of protein was confirmed by staining protein using ponceau red stain for 10 minutes. The membrane was scanned to determine protein had transferred, then carefully washed using d H_2O to remove the ponceau red fig 2.8

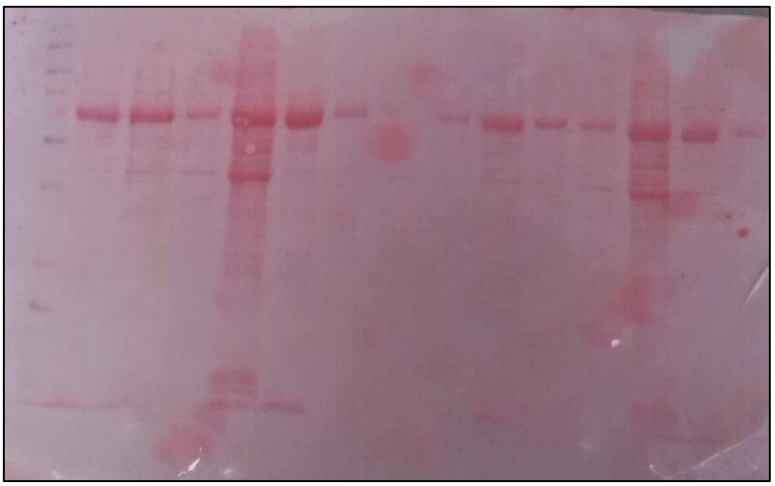


Figure 2.8 Ponceau staining of the nitrocellulose membrane after electro-blotting to confirm the transfer process. The red staining shows successful protein separation and transfer from the gel to the nitrocellulose membrane.

2.9.7 Blocking

After removing ponceau stain from the nitrocellulose membrane - it was immersed for 1 hour in a bath of 5% Marvel milk and TBS, consisting of Tris (12.11g), NaCl (146.1g), dH_2O (5L). The aim of this step was to block the binding site region on the nitrocellulose. This will inhibit any interaction between the membrane and further antibody.

2.9.8 Immune blotting (antibodies incubation)

First day: Primary antibody (AKR1C3 antibody) 12-15 ml at 1:1000 concentrations in a 3% marvel solution was added to the membrane and incubated overnight at 4°C with gentle agitation.

Second day:

After 24 hours, the blot was washed with TBS (washing buffer) x10 for 10 minutes; each wash with shaking. Then the blot was incubated with alkaline phosphatase (AP) conjugated secondary antibody at 1:300000 dilution in 3% marvel for 1.5 hours at room temperature, also with gentle agitation. Again, the membrane was washed as described above. In addition, it was washed twice in TBS to remove TBST, which interferes with enhancer. After, it was incubated with 50µl enhancer and 2.5ml substrate solution for 10 minutes (non-isotopic chemiluminescent detection system Immuno-Star-Alkaline Phosphate kit (AP: Biorad).

The reason for using enhancer and substrate is to produce luminescence. Excess substrate/enhancer from the blot is dripped onto a paper towel and placed in plastic. After re-marking the position of the marker in pen on the plastic, chemiluminescent detection was performed.

The alkaline phosphatase method works based on using hydrolase enzyme, which removes phosphate groups to initiate a chemiluminescent reaction; this can be seen directly and photographed using a camera. The Chemi Doc (vers 4.2.1) imaging system was used in this research to see the immune-reactive bands on the membrane to be used for analysis.

2.9.9 Positive and negative controls

Placental tissue lysate was used as a positive control throughout all procedures or stages of WB. As it is confirmed that 17β-HSD type5 (AKR1C3) expresses in adipose tissue. Positive control is designed to verify working efficiency of the antibodies. In addition, lysate of leucocyte cells from human blood was used as a negative control as it is known that leucocytes do not express 17β-HSD (AKR1C3). The reason to use a negative control is to check antibody specificity. False positive results and nonspecific findings can be detected by using negative control. The reasons for using negative and positive controls are because WB is a complex procedure, taking a long time (48 hours), and consists of 5 stages. Therefore, the risk of error is quite high. Mistakes could happen due to inconsistent transfer, protein degradation, and unequal protein loading. Hence, using of positive and negative control is required.

2.9.10 Antibody strip method

After blotting for the protein of interest, the same blot (nitrocellulose) was probed for β-actin. For loading a control antibody, due to its ubiquitous nature, Re-blot was used and the blot was incubated for 10 minutes (ReBlot Plus, Chemicon International). This enables the previously attached antibody to be stripped away through an alkaline-based solution. The blot was then washed in TBS-T (x3) for 10 minutes, and blocked using 5% marvel milk solution for 90 minutes. The blot was then washed two times with TBS-T solution for 1 minute before addition of the β-actin (1:8000 antibody dilutions) in 3% W/V marvel milk/ TBS overnight on a gentle shaker at 4°C.

2.9.11 Data analysis

Loading control is used to test the quality of the sample and the performance of the secondary antibody system. Loading controls are antibodies to house-keeping express at the same level in all most tissue. And it is needed to examine

that the lanes in the gel have been evenly loaded with sample, especially we need to compare the expression of AKR1C3 between non-PCOS and PCOS adipose tissue samples. In addition, loading controls are helpful to check for even transfer from the gel to the membrane across the whole gel. Furthermore, loading controls are required to quantify the amounts of protein in each lane. In this study, antibodies against β-actin protein were used. The software enabled calculation of the molecular weight of the proteins identified; pre-stained molecular weight markers were used as a guide. All loading controls were studied alongside the results, and the exposure was maintained constant for each of the proteins of interest. The Band Analysis tools of Image Lab software version 4.1 (Bio-Rad) were used to determine subtracted density of the bands in all the gels and blots, the average and the intensities of the bands were measured. All WB experiments were repeated at least three times, and both the mean± SEM were documented. Unpaired t-tests were used to compare between two studying groups.

2.10 3T3 L1 cell line culture

The 3T3 -L1 cell line was utilized in initial cell culture experiments. It is a sub-strain of mouse 3T3 fibroblast from clonal isolation. All procedures pertaining to the culturing of the 3T3- cell line were performed in a class 2 biosafety cabinet using aseptic technique.

The aims from primary work on this cell line were to learn and acquire the skills of laboratory work; such as cell culture techniques in term of storage, maintenance and passaging, as well as observation of cell growth - To then be applied on human cell culture.

2.10.1 Thawing and freezing of 3T3-L1 cell line

The culture medium used for 3T3-L1 cells was (Dulbecco's Modified Eagles Medium) (DMEM) supplemented with 10% foetal bovine serum (FBS), and 1% penicillin (100U/ML)/streptomycin (100U/ml), HEPES (15 Mm).

The frozen 3T3-L1 cells that had been stored in a mix of 1% dimethyl sulphoxide (DMSO) were taken from liquid nitrogen storage and quickly defrosted by placing in a water bath (37°C) for about 1 minute with close observation of defrosting; then cells were gently removed from the

freezing vial using a sterile pipette, and quickly re-suspended by placing in 25 cm2 flask containing 5ml of pre-warmed culture medium - allowing the medium to cover the bottom of the flask.

After labelling, the flask was placed in a humidified 5% CO2/air incubator at 37 °C. Following 24 hrs incubation, culture media was changed. Thereafter every 48hrs, culture medium was changed until confluence was reached. Then the cells were passaged further in a T75 flask for cell expansion. This will be discussed in detail in the next section.

2.10.2 Cryopreservation

Cell suspension was transferred to the labelled cryotubes and 10% DMSO added gradually; the tube was immediately placed on ice for 20 minutes to allow its temperature to decrease by 1°C every minute. The tube containing the cells was transferred from the ice and placed in a cryo-freezing vessel containing isopropanol and placed in the freezer at -80 °C overnight. On the following day, the vial was transferred to liquid nitrogen storage. Content with location were documented for future reference.

2.11 Subcutaneous human adipose primary cells

For several years, primary pre-adipocyte cultures have been successfully developed from mature adipose tissue (Hausman and Poulos 2004). Pre-adipocytes were successfully isolated from SC tissue of women with PCOS and from normal women, in preparation to be used as a model system for subsequent work.

2.11.1 Isolation of pre-adipocytes from fresh adipose tissue samples, primary cultures and maintenance

Fresh adipose tissues explanted after collection and transferred from the operating theatre to the laboratory (**see section 2.1.2**) were kept at 4°C. The procedure used to isolate different cell types from adipose tissue is based on a modified version of the Rodbell method (Rodbell 1965).

All procedures were performed in a biosafety Class II cabinet. Adipose tissue (~5g) was placed in a petri dish (10cm) containing 3-5 ml of 2mg/ml collagenase type 1 made up in HBSS and dissected into small pieces (3-4mm) using a scalpel. At the same time, removing any visible blood vessels and connective tissue.

Small pieces of tissue were then placed in a 50ml Falcon tube containing collagenase solution, and incubated at 37°C for 30-45 minutes in a water bath.

During this period, every 15 minutes the sample was gently triturated with a sterile Pasteur pipette to ensure complete digestion of the tissue. by intermittent checking by placing a small drop on a slide and observing under light microscopy for single cells (**fig 2.9**).

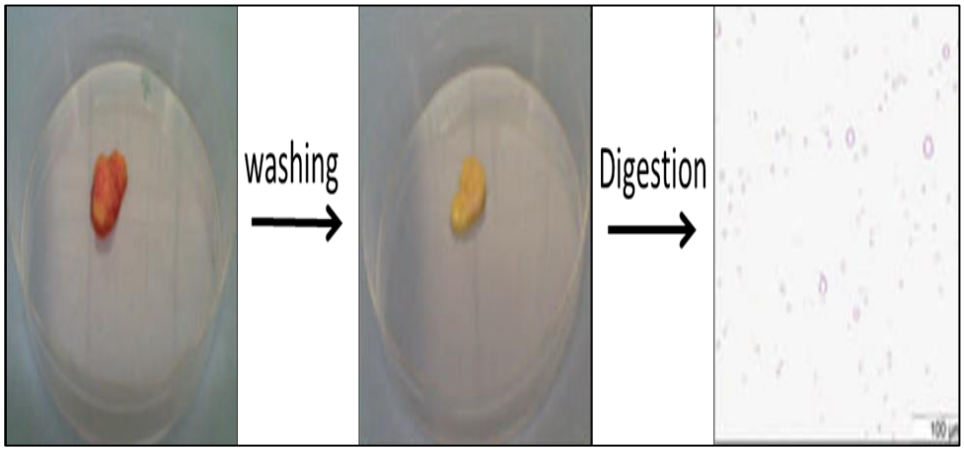


Figure 2.9 Isolation of pre-adipocytes from fresh adipose tissue samples by the enzyme digestion method. After collection and washing, the sample was digested using collagenase, which produced pre-adipocytes.

After dissociation of the tissue, the solution was filtered through a 250μm nylon mesh, resulting in a solution with two phases. The upper phase contains mainly mature adipocytes+lipid, and the lower phase contains stromal vascular cells, including pre-adipocytes.

The lower phase was removed and placed in a separate 14ml tube. The tube was centrifuged at 1000 rpm for 5 minutes; after centrifugation, pre-adipocytes were collected and re-suspended in DMEM in a separate T25cm^2 flask. Cells were incubated at 37°C in a 5% CO_2/air humidified incubator, maintained and split (**fig 2.13**). In fact, it is difficult to distinguish between pre-adipocytes and any cell type with a fibroblast-like morphology; however, the only widely accepted marker of pre-adipocytes is pre-adipocytes factor 1 which is highly expressed in pre-adipocytes (Villena 2002)

2.11.1.1 Maintenance and trypsinisation

Pre-adipocytes were maintained in a high glucose (DMEM) culture medium. Hanks Buffer Salt Solution supplemented with 10nM HEPES and (HBSS 1%/Pencillin/streptomycin). Exhausted medium is recognised by change in the colour of the media from red to orange. This change reflects the presences of several cells on the plate and they are consuming all of the substrates from the media and producing acidic products leading to colour change of the media, and in this case the media needs to be changed and the cells split. Cultures were routinely examined under phase-contrast microscopy to check for contamination and the general health of the cells. Cells were fed every 3 days to maintain them, until confluent. After confluence, the cells for further propagation.

Exhausted medium was aspirated from the cell monolayer. Cells were rinsed with HBSS, and then treated with 1-2ml of 0.25% trypsin–EDTA after being defrosted. Enough enzyme solution was added to cover the cells when the culture vessel was tilted, and was left on the cells for no more than 30 seconds. The flask was then shaken and tapped thoroughly to detach the cells. Two ml of fresh medium containing serum - which inhibits the action of trypsin - was

added. The cell suspension was transferred to a 14 ml Falcon tube and centrifuged at <1000 RPM for 5 minutes at room temperature (22-25°C); the supernatant was then aspirated and the pellet re-suspended in 10 ml of HBSS and centrifuged at 1000 rpm for 5 minutes.

Thereafter, the supernatant was removed, and the pellet prepared for counting by adding 1 ml of medium. Counting was performed using an electrical counter or haemocytometer. The viability of the cells was also investigated by adding 100µl of 10% trypan blue and checking dye uptake into cells.

Using a haemocytometer, cell counting was performed as follows: a cover slip was placed on the middle of the haemocytometer; cells were then diluted to the recommended seeding concentration, i.e. 10µl of cell suspension +90 µl of HBSS to give 100 µL (1:10). 10µl of previous mixture was loaded onto the side of the cover slip by allowing a drop held at the end of the tip to be taken under the slide by capillary action. The haemocytometer was immediately

placed on the stage of a microscope. Grids were located at low power and the cells counted in any one of the 1mm square areas.

The cells were counted in a total of four different 1mm squares and divided by 4 to get an average number of cells/square. After counting, the cells were placed in the T-flasks or well-plate and a suitable volume of medium added (which depends on the size of the flask (5-25 mls); and the cell culture was placed in the incubator at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every 2-3 days (48-72hr) to maintain cells and splitting of cells was carried out when cells were 70-80% confluent.

2.11.2 *In vitro* adipogenesis (adipogenic differentiation)

In addition to normal constituents of culture media for maintaining cell cultures; *in vitro* differentiation requires the addition of a hormonal cocktail including insulin (50nM), Isobutylmethylxanthine (IBMX) (0.25mM), and dexamethasone (100nM); all these reagents were purchased from Sigma Aldrich (UK). Cells from the flask were seeded at a density of 3×10^5 cells/ml in a 24 well plate, and maintained in culture medium at 37°C in a 5% air CO_2

environment until confluent (80-100%), which took about 4-6 days. Medium was changed every 2 days without trypsinisation.

Induction of differentiation was performed when confluence of cells was observed. The culture Induction Differentiation Medium (IDM), which contains the induction hormonal cocktail, was added to cover the monolayer cell; this time point was considered as Day 0 for the differentiation assay. After exposure to IDM for 48hrs (2 days), the cells were incubated in maintenance differentiation medium (MDM), which is similar to IDM, but with the omission of IBMX.

After exposure to IDM for 48hrs (2 days), the cells were incubated in MDM. Cells were renewed every 3 days with observation of the differentiation process by examination under the microscope and taking photographs. Triacylglycerol droplets became visible on day 5, and cells were fully differentiated into adipocytes by day 14.

2.11.2.1 Intra-cytoplasmic lipid accumulation quantification

After induction of differentiation, to assess this process, and assess differentiation into adipocytes, the cells were stained with Oil Red O. Briefly, cell cultures were taken from the incubator and placed in the fume hood. Medium was removed from each well and washed with 2ml of Phosphate-buffered saline (PBS). Cells were fixed in 2ml of 4% paraformaldehyde (PFA); after addition of PFA, the plate was incubated for 10 minutes at room temperature.

In that time, Oil Red O solution was prepared. A stock solution was prepared by weighing out 300mg (0.3%) of Oil Red O powder and adding it to 100 ml of 100% Isopropanol. The working solution was also prepared in the fume hood, by taking 3 parts of Oil Red O from the stock solution and mixing with 2 parts of d H_2O water, and incubating at room temperature for 10 minutes. The working solution is stable for only 2 hrs. In order to remove any particles, the Oil Red O working solution was filtered through a 0.22µm filter.

For staining, after incubation with fixate for 10 minutes, PFA was removed from each well and discarded according to the chemical waste disposal

procedure. Each well was washed with 2ml d H_2O. Then 2 ml of 60% isopropanol was added to each well and left for 5 minutes, isopropanol was removed and 2 ml 5% working solution of Oil Red O was added, and then incubated at room temperature for 5 minutes.

Oil Red O was then removed and cells rinsed with tap water until the water was clear. 2 ml of (1%) haematoxylin stain was added to each well in order to stain the nucleus, and cells incubated for 1 minute at room temperature.

Assessment and measurement: Examination and assessment was performed under the microscope. In order to quantify the extent of differentiation amongst cultures, Oil red O retained by lipid droplets was eluted with isopropanol containing 4% Igebla CA-630. Optical density was measured at 490nm using the spectrophotometer. 200µl of solution was loaded in a 96 well plate.

Alternative and potentially more accurate measures of the differentiation process are q-RT-PCR of adipogenic related transcripts PPAR-γ - which is a nuclear receptor; and fatty acid binding protein transcriptase (FABP4) - a late marker of adipogenesis. In addition, flow cytometry enables the study of differentiation at single cell, accumulation of FABP4 protein molecular marker of adipogenic.

As mentioned above, Oil red dye was used in this study; a disadvantage of this method is it did not give any indication about the cell number or average of lipid per cells; in addition, the absorbance of samples is subject to variation caused by degradation of isopropanol over time. Visual analysis using oil red staining showed heterogeneity within the adipogenesis with a subset of the cells failing to differentiate into fat cells.

2.12 Preparation of cell cultures for hormonal assays

In vitro differentiated adipocytes on day 14 were kept for 24hrs in serum-free medium, in order to remove any hormonal contribution from the hormones in the serum of the medium. Subsequently, the cells were ready to be used in

endocrine assays in order to investigate the action of different treatments: insulin, LH and PI3-Kase inhibitor (LY294002) on expression of sex steroidogenic enzymes (androgens) using the qRT-PCR technique, and on testosterone production from adipocytes using ELISA techniques.

Fig 2.11 illustrates the steps in the preparation of already differentiated cells for hormonal assays, which include RT-PCR and ELISA. The aims from the hormonal assays were:

Investigating the action of insulin at different concentrations (1, 10, and 100 nm/l) on the expression of the two study genes using RT-PCR. Also, on the production of testosterone hormone from adipocytes using ELISA. It is important to detect that the response of adipocyte to insulin stimulation to be correspond to *in vivo* results, therefore, based on published data we chose insulin concentrations between 1 and 100 nm/l. 1-10nm/l is within the physiological range and is consistent with insulin actions mediated by insulin receptor. 100 nm/l is a high concentration of insulin (supra-physiological concentration). Based on previous published data which confirm that insulin at physiological level stimulates ovarian androgen production (Nestler 2005) and even at supra-physiological concentrations, insulin effect through insulin receptor (Nestler 2005) (Franks 1999).

- Studying the effect of LH, at concentration of 10nm/l, also due to incubating it with adipocytes, for the same reasons. Correspond to physiological concentration of LH
- The action of insulin +LH were investigated by incubating the cells with insulin (1-100) and LH (10).
- All the above treatments (insulin and LH) were tested under a PI3 Kinase inhibitor, using LY294002 treatment.

After passaging of the primary cells and before seeding in 24 well plates, the viability of the cells was examined using trypan blue exclusion.

After that, at 14-day differentiation, the cell culture was treated with the above mentioned treatment (insulin, LH, and LY294002). At a relevant time

point (72hrs), as based data in the literature indicate that induction of insulin of enzyme activity, 17α-hydroxylase is time dependent and insulin stimulates enzyme activity at 72hr (Munir 2004).

The conditioned medium was removed and stored at -80°C to be used later for steroid measurements.

In addition, for gene analysis study, RNA was extracted from the monolayer cell culture using the Trizol reagent method. This will be discussed later, and is to be used to study the action of above treatments on the expression of the two study genes, using the qRT-PCR technique.

Treatment of *in vitro* differentiated adipocytes

EXPERIMENTAL DESIGN

Adipose tissue biopsy → Freezing at -80 °C

↓

Isolation of pre-adipocytes from fresh biopsy by enzyme digest

↓

Culture and maintenance of the cells in MM until confluence, **THIS TAKES ABOUT 10-14 DAYS**

↓

Splitting with Trypsin and seeding the cell in 75 T (2-3) flask for expanding, until confluent, **THIS TAKES 10-14 DAYS**

↓

Splitting, and seeding in **24 WELLS PLATES, 5X10^3 Cells/ml, 200µl/well**. Will need 2 wells plates for the **time point 72**hours

↓

Grow until confluence (80-100%), **7-10 DAYS**

↓

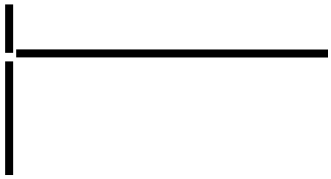
Induction of differentiation with IDM (50nm insulin, 100nm dexamethasone and, 25mm IBMX, cell maintenance in this medium for 48 hrs, after that cell cultured in MDM until differentiated, this **TAKES 2 WEEKS**

↓

Culturing the cell in FCS free medium for **24hr**

↓

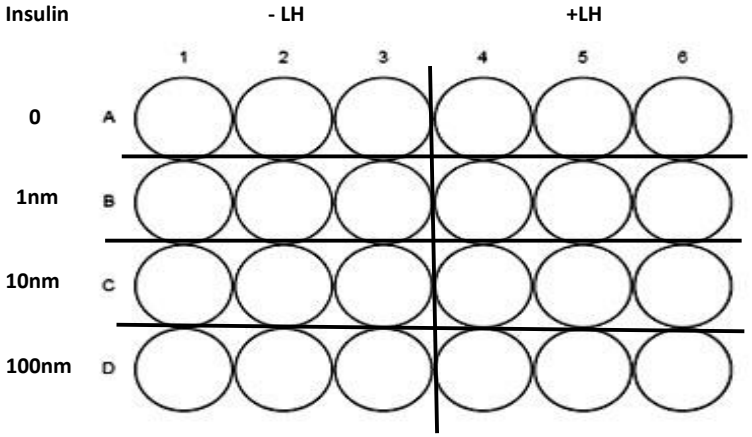
After that start to add treatments (Insulin at different concentration ±LH (10nm/l) and (PI3K+INSULIN) ±LH, at a time point (72hrs)

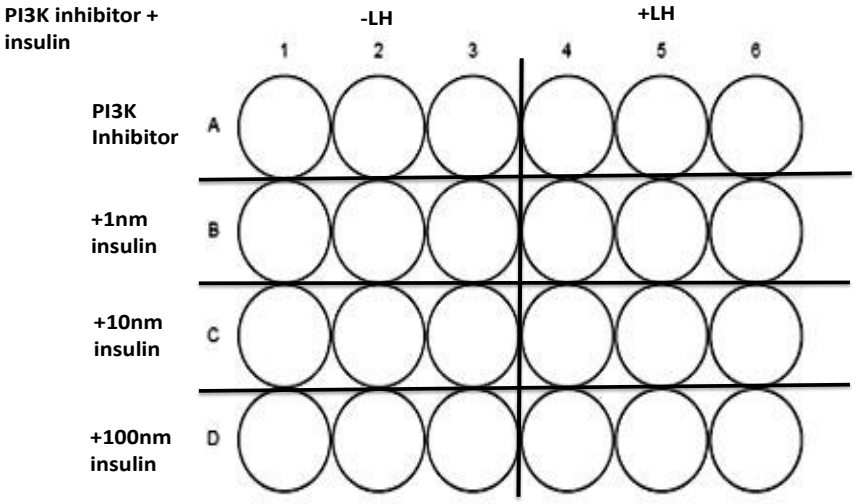


At 72 hrs time point, medium was collected and stored at -80C to be used for **ELISA** to **testosterone hormone** level in medium

On time point (72hrs) cells are lysed with **Trizol** to isolate total RNA from cells for **gene analysis**

Plate design





2.13 Hormonal assay: measurement of testosterone production by ELISA technique

ELISA enzyme immunoassay, also known as an enzyme-linked immunosorbent assay (ELISA), enables the measurement of the concentration of a specific protein in the solution.

There are different types of EIA; a competitive EIA is one of these types, and is most commonly used to measure small molecules such as hormone testosterone in solution. The mechanism of action of this assay is based on the competition between testosterone and a testosterone-acetylcholinesterase (AChE) conjugate (Testosterone Tracer) for a restricted amount of Testosterone Antiserum. The concentration of Testosterone Tracer is constant, whereas the quantity of testosterone in the samples (culture supernatant) varies. Therefore, the amount of Testosterone Tracer able to bind to the Testosterone Antiserum is inversely proportional to the concentration of testosterone in the well. This means fewer tracers will be able to bind to the specific antibody in the presence of greater concentration of analyte or protein of interest (testosterone).

Antigen-Antibody complex (antiserum-testosterone complex) attached to mouse monoclonal anti-rabbit IgG, which was already previously bound to the well. The plate was washed to remove any free (unbound) reagents. After, the Ellman's Reagent was added (this reagent contains the substrates to AChE) to the well.

A distinct yellow colour was seen, which is the production of the enzymatic reaction. The colour intensity was determined using a spectrophotometer, with strong absorbance at 412nm.

The intensity of the colour is proportional to the concentration of Testosterone Tracer attached to the well, which is inversely proportional to the amount of unbound Testosterone present in the well during incubation time.

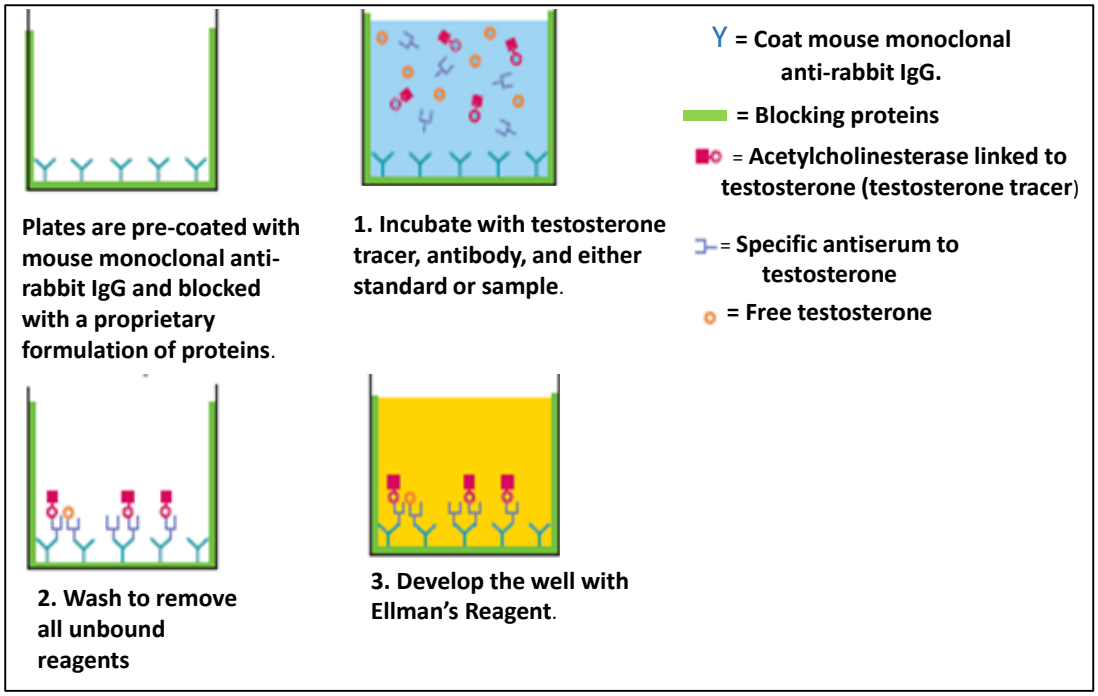


Figure 2.1 Illustration of a competitive ELISA. It shows the steps of detection of the concentration of testosterone hormone in the culture supernatants.

2.13.1 ELISA procedure

ELISA specific plates were used, where 10µl of standard/samples were added to duplicate wells. These also included a blank well to compare and eliminate any background interference. 200µl of testosterone enzyme label was added and left for 2.5 hours at room temperature, covered and gently shaken. Then it was removed and each well was washed (×5) using 200µl of wash buffer for 2 minutes at a time. Then the washing buffer was removed and the wells were tapped dry onto an absorbent tissue. 200µl of alkaline phosphatase substrate was added to each well, and incubated at room temperature for 30-90 minutes. Colour production was directly measured using a spectrophotometer (405-420) and the results were analysed by comparison to the standard.

2.13.2 ELISA standard

Cayman s Testosterone EIA Kit, which has been used in this study, allowed for quantification of samples to a sensitivity of 32 pg/ml, using a standard range of known testosterone sample as supplied. Absorbance was measured (405-420nm) to create a standard curve (**fig 2.13**). The results of the standard curve all fell within an acceptable range (6-32pg/ml) for reliability. All results of the testosterone in the supernatant were calculated using the standard curve after preparation of the data.

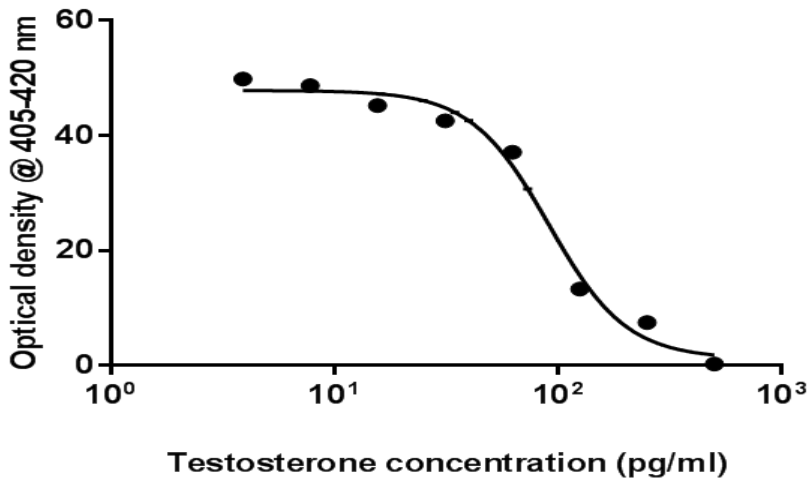


Figure 2.13 Standard curve showing ELISA testosterone levels through calculated absorbance. Known concentrations of testosterone hormone were measured using ELISA technique and the level of absorbance @ 405-420nm was taken to construct the standard curve from which all results were calculated or measured.

2.13.3 Data analysis

All samples were analysed as duplicates, and media blanks were used (DMEM/high glucose). Sample standard was analysed (taken from the ELISA kit), and standard curves created by collating spectrophotometry data within Microsoft Excel 2010. Then the data was transported to Prism software (vers. 5.0b), and interpolated against the standard curves to determine the hormone (testosterone) concentration data (pg/ml). Multiple comparisons of multiple means for unpaired samples were performed using one-way analysis of variance (ANOVA) with post hoc comparisons employing Tukey's test. Statistical significance was considered to be P at or below 0.05.

Unpaired t-test was used when indicated to compare between two populations (PCOS versus non-PCOS). P-values<0.05 allowed rejection of the null hypothesis.

2.14 Statistical analysis

Statistical analysis of data was performed using Graph Pad Prism v5.0 software; the level of P value was set at 0.05. Data are expressed as means± SEM.

Statistical analysis on real-time PCR data was performed on mean delta Ct (ΔCt) values relative comparative of Ct value of target gene relative to the reference genes, Genex software was used. Unpaired t-test procedure was used to compare real time PCR measured expression levels of target genes in SC of PCOS vs NON-PCOS.

Sample size was calculated in order to determine the minimum number of subjects for adequate study power. It was calculated by using Microsoft Excel which contains formulas for calculating sample size and by using http://powerandsamplesize.com/.

CHAPTER 3 RESULTS

3.1 Adipose tissue mRNA expression of steroid metabolising enzymes

3.1.1 Optimisation of RNA extraction method from frozen adipose tissue samples

3.1.1.1 Introduction

The demand to understand the biology of adipose tissue has increased dramatically as the significance of adipose tissue in the human body has changed. Previously it was considered a passive energy tissue, with no active metabolic function (Hauner 2004). Nowadays, it is accepted as an active metabolic and endocrine organ (Avram, Avram et al. 2005), producing hormones and factors which have a very important role in the regulation and control of cell turnover and function; not just at the level of adipose tissue, but also in multiple organ systems such as brain (Azziz, Carmina et al. 2009). The endocrine function of adipose tissue is dependent on size, number of adipocytes (volume, morphology), and on the topography (visceral vs. SC). Moreover, it's based on the characterisers of adipose tissue, which may be changed by genetic and acquired factors (Lacinova, Dolinkova et al. 2008).

Gene expression is controlled at the transcriptional and post-transcriptional level; therefore, the most important method to assess such activity is by determination of the level of corresponding mRNA (Brockmann, Beyer et al. 2007).

The analysis of the gene encoding mRNA of interest is normally performed by a sensitive technique of real-time reverse transcription–polymerase chain reaction (RT-PCR) (Rebouças, Costa et al. 2013).

Successful and reliable use of such a technique of molecular biology needs a sufficient amount of high-quality RNA isolated from the tissue. Using low quality RNA may strongly compromise the end result of the downstream application. RT-PCR is labour-intensive, time consuming and highly expensive (Lacinova, Dolinkova et al. 2008).

RNA is a single stranded structure acting as the intermediate in the flow of information from gene to protein. However, RNA is a versatile molecule involved in a wide range of cellular process from DNA replication to protein synthesis. In comparison with DNA structure, RNA has greater structural versatility, where it is folded into a three-dimensional structure which acts as a globular protein (Lizabeth, A et al. 2012).

Despite the increasing interest in studying the changes of gene expression in adipose tissue, few methodological researches concentrating on RNA extraction from adipose tissue have been published (Tavangar, Hoffman et al. 1990). In general, the most well-known methods to isolate RNA from tissue are solid phase/glass filter methodology, magnetic bead method and organic extraction phenol-based method (Ambion.com), which utilises Trizol reagent.

In this study, the Trizol reagent based method has been used; it is a complete and ready to use technique. Since it was used in 1987, the acid quanidinium thiocyanate-phenol-chloroform has been the method of choice for the extraction of good quality RNA (Chomczynski and Sacchi 1987).

The key advantages of this method are Trizol locks proteins, including nuclease into the interphase and organic phase, enabling clean transfer of the RNA contained in the aqueous phase. In addition, it is a simple procedure allowing processing of a large number of samples at the same time. Moreover, it can be used with small as well as large quantities of tissue and cells. However, obtaining ungraded RNA of sufficient yield and purity needs experience and is time consuming.

In addition, the toxicity of a reagent using quanindin and chloroform has to be considered. In addition to the disadvantages of the procedures, the isolation of RNA from adipose tissue is associated with various problems, which come from specific chemical and biological features of fat tissue ((Janke, Engeli et al. 2001).

The present work was designed to validate and optimise the Trizol reagent method to allow the isolation of total RNA from human small SAT samples in quantities and quality sufficient for application, including RT-PCR, as a cost-effective alternative to commercially available kits.

3.1.1.2 Objectives

The objective is to maximise the Trizol reagent method to isolate total RNA from frozen SAT samples of high quality to be suitable for analysis.

3.1.1.3 Methods

Methods were described in detail in chapter 2, (**see section 2.2**).

- **Statistical analysis**

Mean RNA yield (ng/µl) and purity (A 260/280 ratio) were calculated and compared using one–way ANOVA analysis, and unpaired t-test of the variances. The level of significance was set at $P<0.05$. Mean and SEM are presented. Graph-pad Prism version 6 software was used.

3.1.1.4 Results

3.1.1.4.1 Determination of the starting weight of adipose tissue

We detected the maximal amount of adipose tissue, which can be used in the procedures without effect on the resulting RNA. We found that 120mg - 130mg of frozen SAT samples is optimal for use to obtain RNA with good concentration and purity - suitable to be used in PCR.

This study found that there is a highly significant relationship ($p < 0.0001$) between the weight of tissue sample and the concentration of extracted RNA. **Fig. 3.1** shows the relationship between RNA yield and amount of adipose tissue. RNA concentration gradually increases with increasing sample weight from 100mg to 140mg. However, after that, the RNA quantity or concentration decreased with rising sample amount. This is related to the decrease in the efficiency of Trizol reagent with increases in the weight of the adipose tissue sample. By following the commercial instruction for 100mg of tissue sample we have to apply 1ml Trizol reagent. However, based on the nature of adipose tissue which contains mainly fat, we increased the weight of the sample gradually without parallel increases in the reagent volume in order to avoid the toxicity of the reagent. We found after 130 mg, the ability of Trizol reagent at 1ml concentration decrease. **Table 3.1.**

Weight sample (mg)	RNA concentration(ng/µl) Mean ±SEM	P value
100mg-120mg	59.52±4.6- 384.4±60.7	0.0004
100mg-130mg	59.52±4.6- 455.4±52.6	0.0001
100mg-140mg	59.52±4.6- 283.3±61.1	0.03
110mg-120mg	119.0±40.6- 384.4±60.7	0.02
110mg-130mg	119.0±40.6- 455.4±52.6	0.001
130mg-150mg	455.4±52.6- 246.8±44.5	0.03

Table 3.1 shows the relationship between RNA concentrations (ng/µl) and weight (mg) of frozen SAT samples of non-PCOS. RNA was isolated by using Trizol reagent method and its concentration was measured using a NanoDrop spectrophotometer based on the absorbance at 260nm. Mean and SEM of RNA yield (ng/µl) were calculated and compared using one-way ANOVA analysis. The level of significance was set at $P<0.05$.

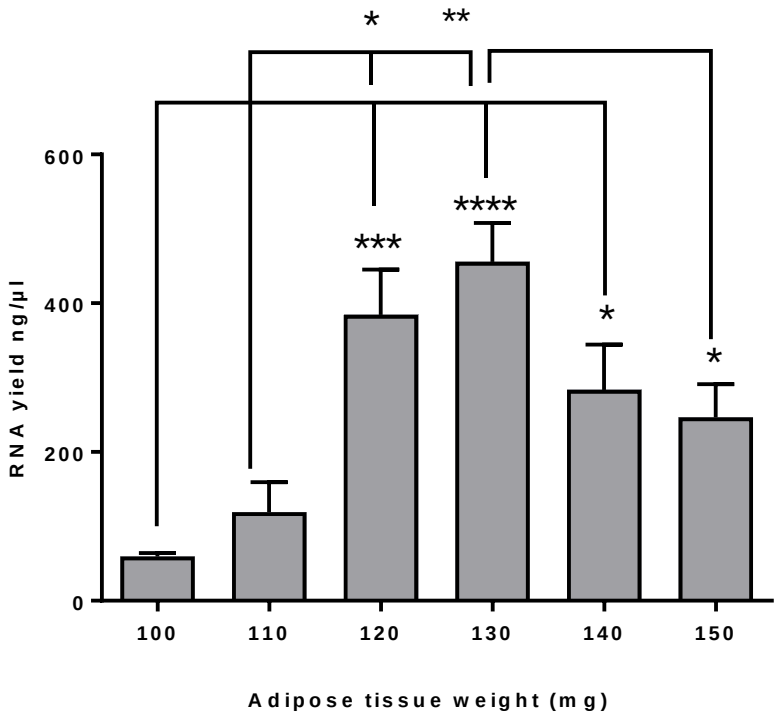


Figure 3.1 Relationship between the concentration of isolated total RNA (ng/µl) and weight (mg) of frozen SAT samples. Total RNA was isolated by Trizol reagent procedure, and the quantity was reported based on the absorbance at 260nm using a NanoDrop spectrophotometer. There was a highly significant relationship between weight of the tissue samples and RNA yield **** P <0.0001. RNA concentration gradually increased with increases in the weight of tissue samples (n=40) from 100 mg to 130mg, but after that, the RNA yield decreased with increases in the weight of tissue samples of 140mg and 150mg. Results of experiments were expressed as mean RNA yield (ng/µl) and SEM, mean RNA concentration was calculated, and multiple comparisons were performed using one-way ANOVA with post hoc comparisons employing Tukey's test. Statistical significance was considered to be P at or below 0.05. *P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001.

In addition to the concentration, this study detected that there is a significant relationship between weight of tissue samples and purity of extracted RNA ($p<0.0001$), purity decreases with increases in the weight of tissue samples **(Table 3.2)** (**fig. 3.2**).

Adipose tissue samples weight (mg)	RNA purity ($A_{260/280}$) mean± SEM	P value
100mg-120mg	1.47±0.06-1.76±0.04	0.001
100mg-130mg	1.47±0.07-1.81±0.02	0.0001
110mg-120mg	1.38±0.06-1.76±0.04	0.0004
110mg-130mg	1.38±0.06-1.81±0.02	0.0001
130mg-140mg	1.81±0.02-1.54±0.04	0.008
130mg-150mg	1.81±0.02-1.57±0.01	0.005

Table 3.2 shows the relationship between adipose tissue samples (n=40) weight (mg) and the purity of extracted RNA (A 260/280) ratio.

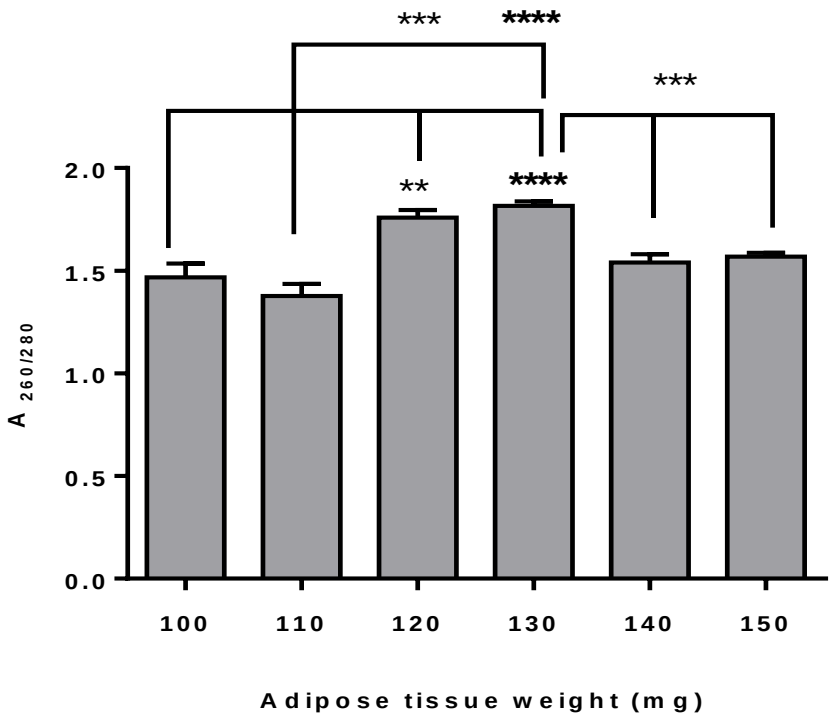


Figure 3.2 demonstrates the purity of extracted total RNA from differently sized frozen SAT samples. The purity of extracted RNA using Trizol method was reported as the ratio of the absorbance at 260/280 using a NanoDrop spectrophotometer. Each value is reported as mean ±SEM. * P<0.05, ** P <0.01, *** P <0.001, **** P<0.0001.

3.1.1.4.2 Optimisation of homogenisation steps

This study explored the significance of the optimisation of the homogenisation step in RNA extraction, and the importance of this step in RNA extraction. The homogenisation step of RNA isolation was validated by mincing the tissue in cold Trizol reagent into small fragments with a scalpel before being homogenised by a mechanical homogeniser. Furthermore, the outcome of the procedure was significantly improved by removing lipid from the homogenate by centrifuging the sample after homogenisation, and before the phase separation step. In this step, the fat layer is eliminated, removing fatty acids in the adipose tissue sample, which interfere with the precipitation of RNA, and so the result of the method improved. **Figure 3.3** shows the optimisation of this step in the procedure.

The result of the procedure in terms of quantity and purity was improved significantly ($P<0.0001$) ($P<0.0001$) with optimization of the homogenisation step of RNA extraction, **table 3.3**. **Fig 3.4** Shows RNA yield ng/µl**, fig 3.5** Shows RNA purity (A 260 /280) before and after optimization of the homogenisation step of RNA extraction.

Extracted RNA was considered good quantity if RNA yield was >300ng/µl (Invitrogen), and pure if A 260/280 ≥1.8 or low purity if A 260/280 < 1.8. Data represent mean± SEM, ****P <0.0001.

	Before optimization	After optimization	P value
RNA concentration (ng/µl) mean± SEM	184.4±27.55	461.7±30.67	<0.0001
RNA purity ($A_{260/280}$) mean± SEM	1.53±0.03	1.82±0.01	<0.0001

Table 3.3 shows the significance of optimization of the homogenisation step on RNA results in terms of concentration and purity. The mean± SEM of RNA concentration ng/µl and RNA purity A 260/280 ratio before and after optimization. Statistical analyses were performed using paired t-test because only adipose tissue samples of non –PCOS women (n=20) were compared before and after optimization.

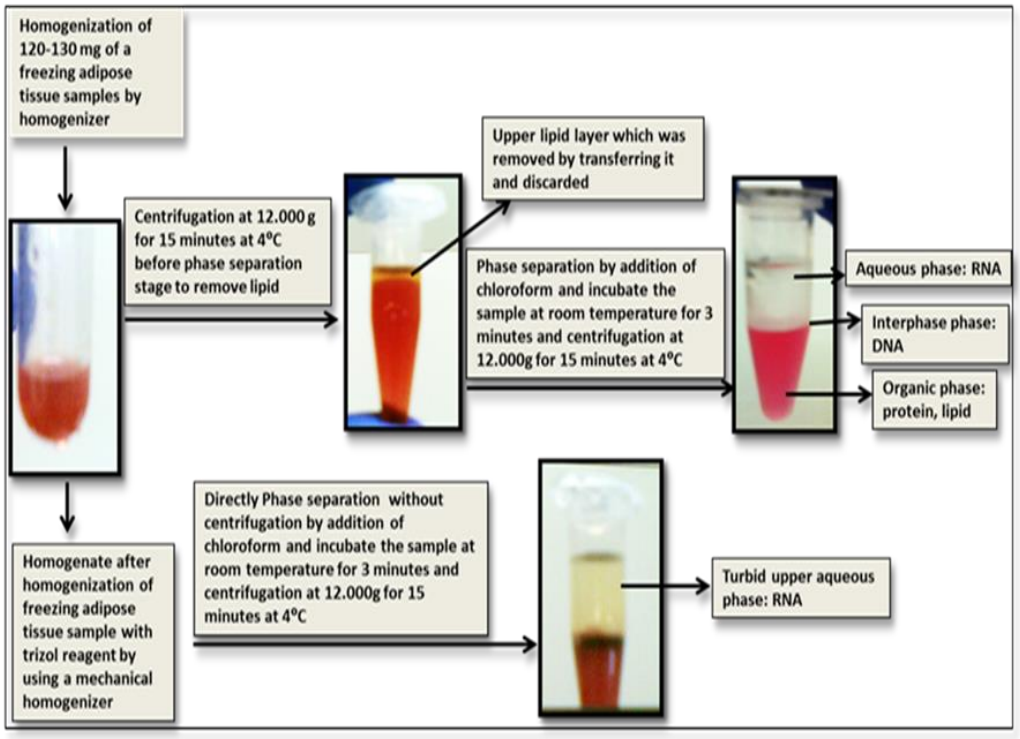


Figure 3.3 schematic demonstrates optimisation of homogenisation step. It describes the separation of the mixture into three layers by centrifugation after addition of chloroform to the sample after and before lipid removal. The adipose tissue sample was homogenised by addition of 1ml of Trizol reagent for each 120-130 mg using a mechanical homogeniser. In order to remove lipid, which interferes with RNA precipitation, the homogenate was centrifuged after being transferred to an Eppendorf tube at 12.000xg for 15 minutes at 4 °C before adding chloroform for phase separation. After centrifugation, the upper lipid layer was removed and discarded - .0.2 ml of chloroform was added, and the sample incubated at room temperature for 3 minutes. Subsequently, the sample was centrifuged at 12.000xg for 15 minutes at 4 °C. After centrifugation, three layers were seen (i) A top aqueous clear layer where the RNA is suspended, the volume of this layer is 60% of volume of Trizol; (ii) a middle interphase layer where DNA is found; and (iii) a lower red organic phase where proteins and lipid are present. However, if the lipid is not removed from the sample, an upper turbid layer is commonly seen.

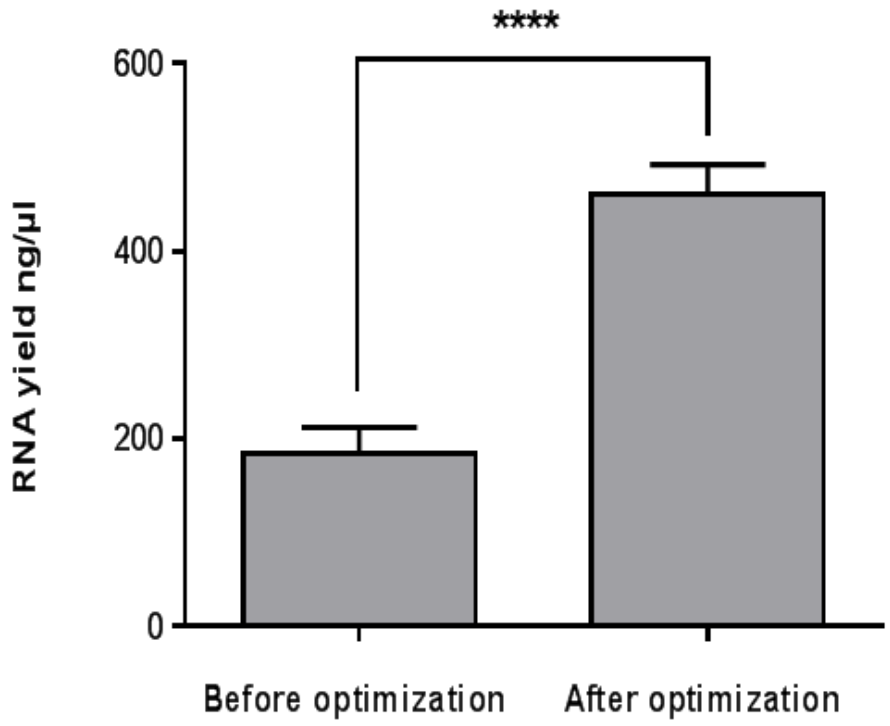


Figure 3.3 shows the improvement in the quantity of RNA yield (ng/µl) after optimisation of the homogenisation. The homogenisation step of frozen fat samples was optimised by mincing a tissue sample in cool Trizol within a short period. Subsequently, removing lipid from homogenates before homogenising using a mechanical homogeniser, and then optimised by centrifuging the homogenate to remove lipid, which interferes with the precipitation of RNA, before addition of chloroform for phase separation. Data are presented as mean±SEM. Paired t -test was used to compare between RNA results of non-PCOS adipose tissue samples (n=20). P <0.0001.

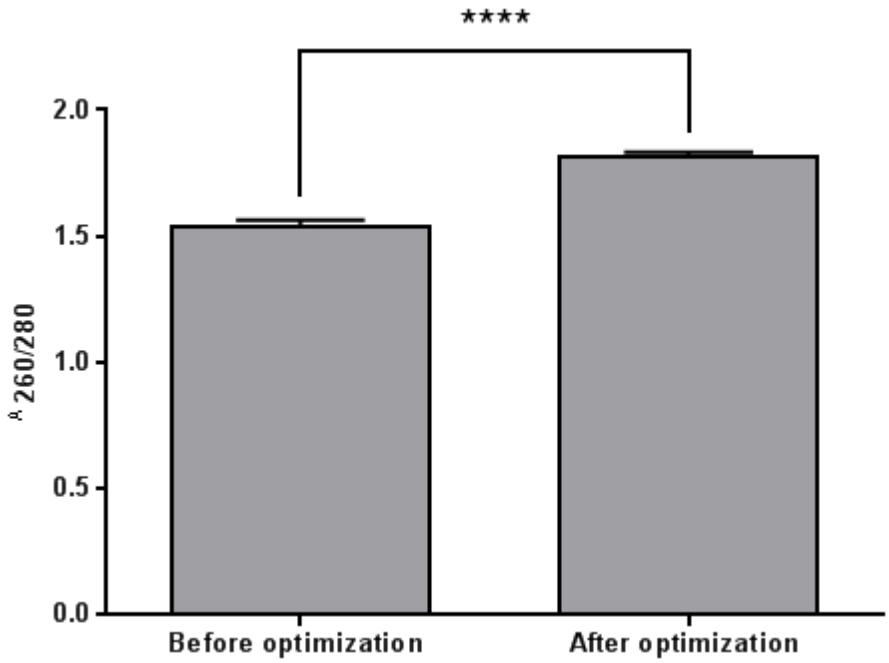


Figure 3.5 shows the purity of isolated RNA before and after optimisation of the homogenisation step. Optimisation step is described in the text.

3.1.1.4.3 Effect of sample condition on extracted RNA

The physiology and biology of the tissue affects the extracted RNA results, and are considered among the internal factors impacting RNA quality. This study investigated the effect of status of the source of the sample on the extracted RNA. **Fig 3.6 and 3.7** demonstrate that there is a significant relationship (P<0.0001) between the RNA quantity and status of the tissue samples. Also there is a significant relationship (P=0.001) between status of the sample and purity of extracted RNA, **table 3.4**. This may reflect the difference in the biology of adipose tissue samples of women with and without PCOS.

	RNA yield (ng/µl) mean± SEM	RNA purity (A260/280) mean± SEM	P value
Non-PCOS (n=14)	440.2 ± 32.36	1.776±0.019	<0.0001
PCOS (n=10)	166.1 ± 20.32	1.658±0.027	0.001

Table 3.4 demonstrates the relationship between the status of the source of the sample and the RNA results in terms of quality and purity. The status of the source of adipose tissue samples has a significant effect on the RNA concentration and purity.

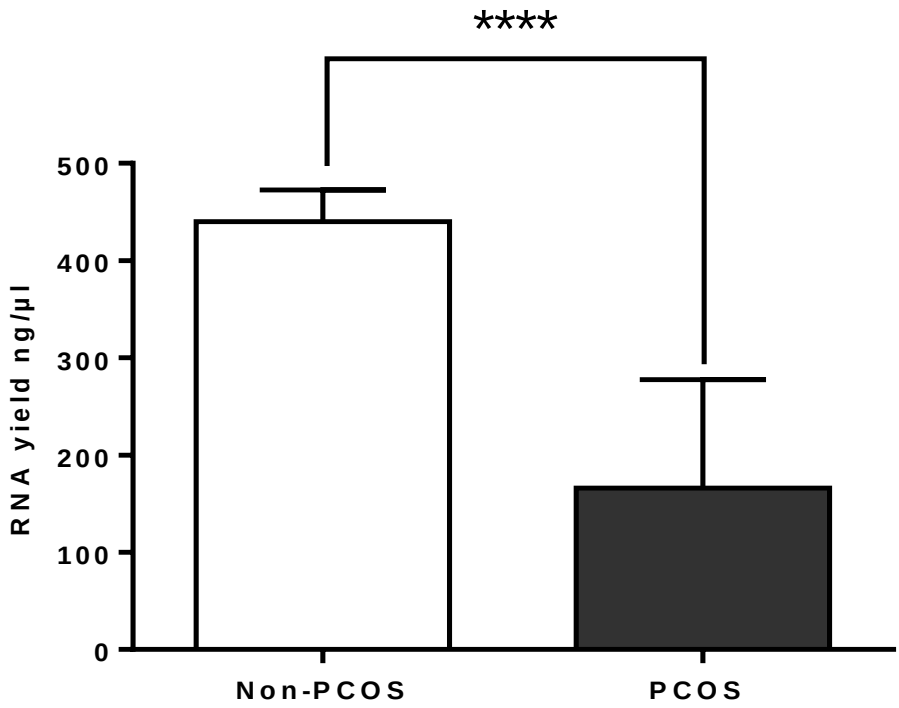


Figure 3.6 shows the effect of sample status (non-PCOS, PCOS) on the concentration of the extracted total RNA from adipose tissue samples of non-PCOS (n=14) and PCOS (n=10) women. RNA quantity (ng/µl) was assessed using a Nano Drop spectrophotometer based on absorption at 260nm. The data were analysed using un-paired t-test to compare between two groups. Data are presented as mean± SEM. **** P<0.0001

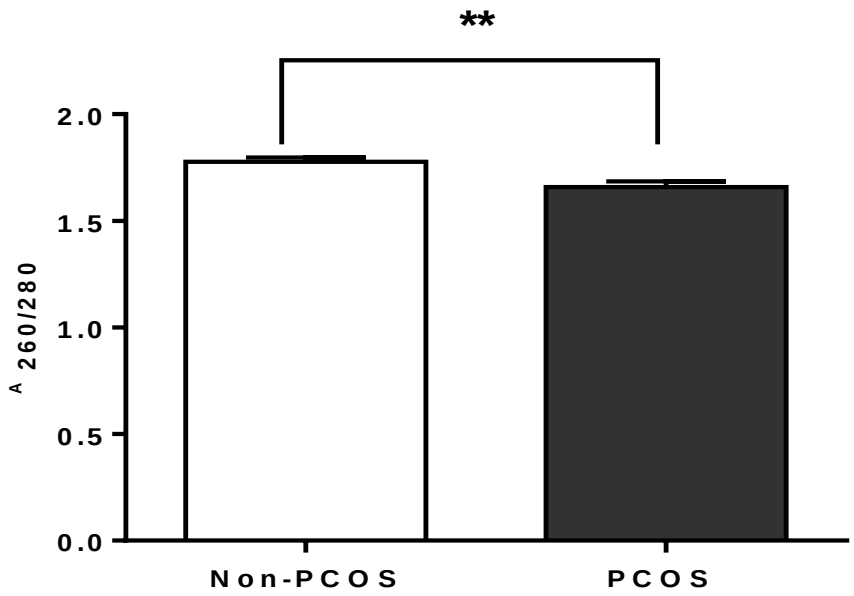


Figure 3.7 shows the relationship between the purity of the extracted total RNA and the status of the source of samples (healthy women without PCOS (n=14), and women with PCOS (n=10)). The purity of isolated RNA was evaluated using a NanoDrop spectrophotometer, the measurement was based on the ratio A 260/280 nm. The data are reported as mean± SEM. ** P =0.001

3.1.1.4.4 Assessment of extracted RNA

Optimisation of the procedure of total RNA extraction from adipose tissue was assessed, using Nano-Drop Spectrophotometry and agarose gel electrophoresis.

3.1.1.4.4.1 Quantity and Purity of isolated RNA

Assessment of the quantity and purity of extracted RNA using conventional methods such as Nano-Drop is often not sensitive and not specific for single-stranded RNA. However, using a UV-spectrophotometer characterises at multiple wave lengths: at 240nm (background absorbance), 260nm (specific for nucleic acid), and 280nm (specific for proteins). Based on the OD 260 the quantity and, by the ratio of the optical density (OD) of $A_{260/280}$ for the purity (Imbeaud et al., 2005), the performance of the extraction technique can be evaluated.

Quantity and purity of RNA extracted from adipose tissue taken from non-PCOS and PCOS women. The concentration was within the reliable range of the NanoDrop ND-1000 (10.0ng/µl and 3700ng/µl), RNA with high purity is close to ideal A260/280 ratio, which indicates no presence of contamination like proteins.

3.1.1.4.4.2 Integrity of isolated RNA

To assess the integrity of extracted RNA from adipose tissue samples - gel electrophoresis was used. This method of RNA integrity assessment is one of the most common techniques used. The results from the gel electrophoresis from the non-PCOS samples before and after optimisation of RNA extraction procedure are shown in the figure 3.8. It is clear that the results explore the significant of optimisation of method to obtain RNA of good quality in term of integrity. Integrity of isolated RNA can be demonstrated in gel electrophoresis by detecting intact ungraded bands at two different positions, i.e. 4.8 kb and 1.8 kb, which represent 28S and 18S ribosomal RNA subunits, respectively **fig 3.8.**

Fig 3.9 shows the significance of optimisation of RNA extraction procedures on the quality of isolated RNA. Integrity of extracted RNA is one of the most important characters of RNA to be used for next step of gene analysis, which is RT-PCR. Before optimisation of RNA extraction by Trizol reagent method, there was difficulty to obtain RNA with good quality in terms of integrity. Most RNA obtained was degraded and contaminated with DNA, this is not suitable to be used for studying gene expression by PCR technique. However, after optimisation of the technique of RNA extraction from adipose tissue samples as mentioned above **see section 2.3.2** we obtained intact RNA, good quality and suitable to be used for gene analysis investigation.

Similarly, to explore the integrity of the RNA extracted from adipose tissue samples by using optimised methods of RNA extraction as mentioned above, the gel electrophoresis technique has been used. As a result, for the purposes of making cDNA, only samples with good quality RNA in terms of integrity were taken into consideration

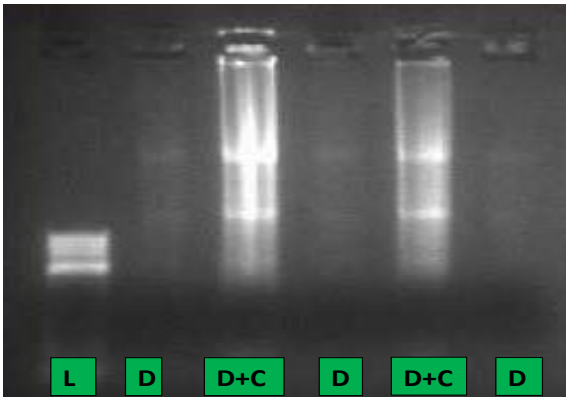


Figure 3.8 this figure shows the ultraviolet (UV) exposure of the isolated total RNA from adipose tissue samples of non-PCOS women before optimization of the RNA extraction method on the 1% agarose gel solution. "**L**" stands for 1kb DNA ladder, "**D**" stands for completely degradation invisible RNA, and "**D+ C**" stands for RNA degradation and contamination with DNA. RNA with these results had been disregarded from the analysis due to degradation and/or contamination with DNA

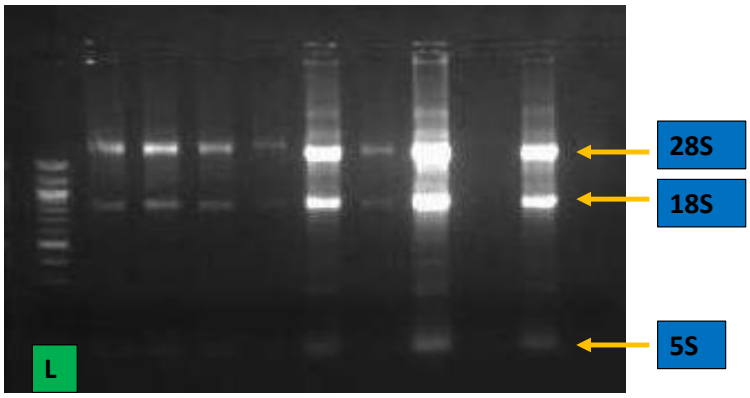


Figure 3.9 shows total RNA isolated from adipose tissue samples of non-PCOS by the optimized RNA extraction method. After optimisation, ungraded intact RNA shows two distinct ribosomal bands, (28 S) and (18S) r RNA and 5S. The 18S and 28S bands are seen, the 28S band being twice as intense compared to the 18S. The lanes represent intact isolated RNA from adipose tissue of women, which appeared as two intense fluorescent clear and well defined bands. "**L**" stands for 1kb DNA ladder**.**

3.1.1.5 Discussion

Several factors affect the results of RNA extraction, and hence, the reproducibility of the relevant results. These factors include the source of RNA, sampling technique, collection, storage, and the RNA isolation method (Nolan, Hands et al. 2006). The researcher must always take into account variation in these factors, which affects their ability to obtain high quality RNA, free from protein and genomic DNA. The other vital issue, which may arise in the study of human tissue, is the difficulty of minimising the sampling technique within the framework; as result, the quality of RNA is impaired. In addition, if the sample is stored for a long time or under-sub-optimal conditions, the risk of impairment of the quality of RNA is high (Wilfinger, Mackey et al. 1997); (Schoor, Weinschenk et al. 2003). In this study, when we started RNA extraction we followed the manufacturer's instructions; however, low yield and highly contaminated RNA was obtained. This confirmed the presence of other factors, which we have to consider when starting extraction.

In fact, optimisation of the technique starts from the time of sampling technique, sample collection and storage until RNA extraction. Properly handling the sample (i.e., using scalpels/scissors to obtain adipose tissue) and clean processing techniques to prevent contamination with RNAase.

The time from specimen collection until flash freezing and storage is important; the sample must be collected within 20 minutes (Cashion, Umberger et al. 2011) and the tissue samples and isolated RNA stored in an ultra-low-temperature freezer - to maintain the tissue structures and biochemical parameters inhibiting enzyme activity and other processes

Our data are in accordance with the finding of the mean RNA concentration obtained from various amounts of starting tissue samples. We have determined that there is a significant relationship between increases in the amount of tissue and RNA quality. We found that 120-130 mg of adipose tissue is optimal for use in the isolation of RNA from frozen SAT samples. With higher amounts of adipose tissue, the yields were low. We suggest that it may be related to the decrease in the efficiency of Trizol reagent, due to increases in the amount

of tissue without a parallel increase in the concentration of the reagent. Based on the instructions of the manufacturer (Invitrogen.com), for every 100mg of tissue we have to apply 1ml of Trizol. However, in this study, we gradually increased the amount of tissue without increasing the reagent volume. This issue can be resolved by increasing the amount of adipose tissue with increases in the concentration of the reagent at the same time. However, this is difficult because the tissue samples are of human adipose tissue, and it is not always easy to obtain a good amount of sample.

The second difficulty that has to be considered is the toxicity of the chemicals, such as phenol and chloroform in the reagent used (Trizol). The long period of exposure, due to long protocol steps, may lead to these impurities (chloroform genomic, and RNase) contaminating the RNA. As such, these chemicals may interfere with RT-PCR. In addition, we have to consider the toxicity on the researcher and workplace, not just the effect on the purity of the samples.

Furthermore, the best RNA yield and purity was obtained from adipose tissue after modification of the homogenisation step. It was modified by mincing the tissue samples into small fragments after submerging in liquid nitrogen, and before homogenising with a mechanical homogeniser. Mincing the tissue samples in cold Trizol before homogenisation enhances the efficiency of the reagent (Invitrogen).

However, Bustin and Nolan (2004) reported best RNA yield when the sample was diced into small parts, before being frozen by submerging it in liquid nitrogen. The reason for avoiding this in this study was to avoid handling of the tissue, and to keep collection time short to protect RNA from internal and external RNase degradation. Hence, the samples were minced after submerging in the liquid nitrogen.

NanoDrop ND-1000 Spectrophotometry (A260) gives accurate and reproducible measurements within the 220-750 nm spectral range, and to measure the concentration and purity it requires only 1 μL of sample. This is one of the key advantages of this method. In addition, it is simple with commonly used equipment. However, it is considerably less sensitive than other methods

that can be used to assess isolated RNA, such as Fluorescent day (Ribo Green) and Agilent 2100 bio analyser 43. In addition, it cannot assess the integrity of extracted RNA when compared with the Agilent 2100 bio analyser, which is not available everywhere (applied bio system 2006). The integrity of the used total RNA should always be evaluated, such that the quality and integrity of RNA have to be preserved, especially in the clinical application, which is characterised with the limitation of tissue material; e.g. obtained during or after surgery. Therefore, careful handling and storage should be practised, as RNA is well known for its sensitivity to degradation through inadequate sample handling or storage (Perez-Novo, Claeys et al. 2005); (Bustin and Nolan 2004).

Moreover, data from this study shows that the quality of RNA extracted from adipose tissue is based not only on the modification of homogenisation of the samples, but also on other specific features of the tissue samples - such as the content of lipid in adipocytes. By removing the fat layer from samples after homogenisation and before phase separation or adding of chloroform, the results dramatically improved in terms of RNA quantity, purity, and integrity.

In addition, the data also demonstrated that the quality of the isolated RNA was based on the background of the tissue samples. There was significant difference in the quality of the RNA extracted from samples with PCOS in comparison with samples taken from women without PCOS. This may be related to the difference in the feature or characteristics of the tissue samples, and in the physiology and pathology of the samples. The size of adipocytes from women with PCOS is greater (Manneras-Holm, Leonhardt et al. 2011). This adipocyte hypertrophy was due to a reduction in lipolysis in the SAT of PCOS women (Ciaraldi 2000); (Faulds, Ryden et al. 2003). In 2001, Janke found that the content of glycerides in adipocytes has an effect on the quality of extracted RNA from adipose tissue, as glycerides interfere with isolation. As mentioned above, the biology of adipose tissue is affected by several acquired and genetic factors (Janke, Engeli et al. 2001).

Our results show that the original Chomczynski and Sacchi RNA isolation procedures (Chomczynski and Sacchi 1987) can be successfully modified by adding and eliminating certain steps. A successful result of RNA

isolation from SAT with the Trizol reagent method was obtained after consideration of all factors that affect the quality of isolated RNA.

Including sampling techniques, sample handling and collection, modification of the technique of extraction are based on the nature of tissue used. As reported here, removal of lipid from adipose tissue samples led to significant improvement in extraction results. Furthermore, the efficiency of the reagent used was enhanced by mincing the tissue in the reagent before homogenisation.

3.1.2 Validation of reference genes in subcutaneous adipose tissue (SAT)

3.1.2.1 Introduction

qRT-PCR is the standard method to study changes in relative gene expression in different types of tissue and under different experimental conditions. This technique is characterised by high sensitivity and specificity (de Jonge, Fehrmann et al. 2007). However, errors in the quantification of gene expression may occur due to several factors, such as variation in the amount of starting material, efficiency of enzymes, and the presence of inhibitors (de Jonge, Fehrmann et al. 2007). Therefore, normalisation of expression data is a critical and vital factor (Vandesompele, De Preter et al. 2002). The most common strategy and approach for data normalisation is the use of reference genes as internal controls (Bustin 2000); (Bustin and Nolan 2004); (de Jonge, Fehrmann et al. 2007).

A typical reference gene is one consistently expressed at the same level in the same cell type or tissue, and reveals expression levels in comparison to those genes under study (Suzuki, Higgins et al. 2000). Based on published data, the most commonly used genes in qRT-PCR studies of adipose tissue are β-Actin (ACTB), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S, Hypoxanthine phosphoribosyl transferase 1 (HPRT1), Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation protein, Zeta polypeptide (YWHAZ), Ubiquitin C (UBC), and low-density lipoprotein (LDL) receptor-related protein 10(LPR10) (Gabrielsson, Olofsson et al. 2005); (de Jonge, Fehrmann et al. 2007). However, multiple studies have shown that the expression of these commonly used reference genes differs with the type and physiological state of the tissue (Thellin, Zorzi et al. 1999); (Bustin and Nolan 2004).

This disparity may lead to misinterpretation of data (Suzuki et al., 2000b). Hence, the suitability of a certain reference gene is based on the system being investigated, and the inherent experimental conditions (Livak and Schmittgen 2001); (Schmittgen and Zakrajsek 2000).

Several recent studies have observed that the expression of reference genes in fat tissue is affected by factors such as obesity and T2DM (Livak (Livak and Schmittgen 2001); (Gabrielsson, Olofsson et al. 2005). In light of these findings, the validation of the stability of the reference genes being used is one of the most important factors for studies of adipose tissue.

Therefore, this study was performed to identify and evaluate the stability of expression of six selected reference genes, which were selected depending on previously published studies that relied on these genes as internal control genes (Catalan, Gomez-Ambrosi et al. 2007); (Gabrielsson, Olofsson et al. 2005); and to determine these housekeeping genes for qRT-PCR experiments using SAT samples taken from a normal healthy non-PCOS group and another consisting of women with PCOS.

3.1.2.2 Objectives

- To select and determine suitable reference genes to be used as internal controls in the gene expression analysis of SAT of women, with and without PCOS, in RT-PCR experiments.

- To evaluate the efficiency of the assay (RT-PCR) by creating standard curves for the reference genes and target genes in order to confirm the optimisation of the assay which will be used for gene expression analysis.

3.1.2.3 Methods

The method employed was described in detail in **Chapter 2 section 6.** Candidate reference genes were tested, namely beta-actin (ACTB), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-2-microglobulin (B2M), hypoxanthine quanin phosphoribosyl guanine phosphoribosyl transferase1 (HPRT1), zetapoly peptide (YWHAZ), and Low-density lipoprotein receptor-related protein 10 (LPR10), all obtained from (Applied Bio-system) (Invitrogen).

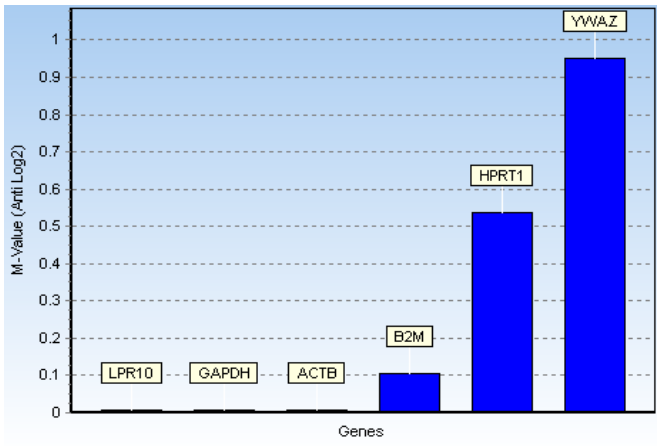
The performance of the assay and generation of standard curves were performed by running serial dilutions consisting of five points of 10-fold serial dilutions starting with 120ng of total RNA concentration generated in triplicate.

Reference gene expression stability across all samples was determined using *GeNorm* and *NormFinder* software from GenEex software performed as described by (Vandesompele, Kubista et al. 2009), (Vandesompele, De Preter et al. 2002), and (Andersen, Jensen et al. 2004). The stability of each gene was calculated using the mean Ct (threshold cycle) values, which is the intersection between an amplification curve and a threshold line (Applied Biosystems guide). The software converted raw Ct values to relative quantities using the comparative Ct method (Vandesompele, De Preter et al. 2002).

3.1.2.4 Results

3.1.2.4.1 Reference gene determination

Reference genes were determined by Genex software. The first analysis used *GeNorm* analysis to compute the stability value of the candidate reference gene (M) using raw non-normalised expression values (Ct). The gene with the lowest expression stability (i.e. the lowest M value) was eliminated. This analysis was continued until only the genes with the lowest M value and most stable expression persisted. **Fig 3.10 A** shows the most stable genes with the lowest M value expressed in adipose tissue of women without PCOS, which are LPR10, GAPDH and ACTB, (M) value 0.005, 0.005, and 0.009 respectively. In women with PCOS (**fig 3.10 B**), LPR10, ACTB, and GAPDH (M) value 0.005, 0.005, and 0.012 respectively. The difference between *GeNorm* and *NormFinder* is described in the discussion, **section 3.1.2.5.**



(A) Gene Name M-Value

YWAZ 0.951

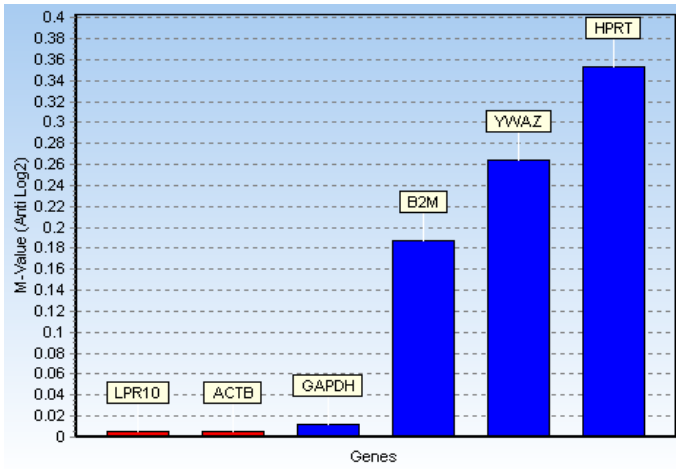
HPRT1 0.538

B2M 0.106

ACTB 0.009

GAPDH 0.005

LPR10 0.005



(B) Gene Name M-Value

HPRT 0.353

YWAZ 0.263

B2M 0.187

GAPDH 0.012

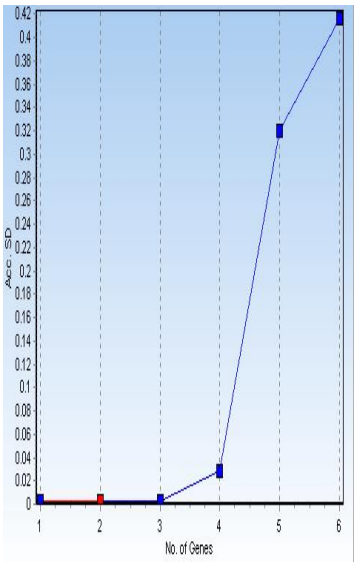
ACTB 0.005

LPR10 0.005

Figure 3.10 Gene expression stability (M) of candidate reference genes in SAT of women (A) without PCOS (n=5) and (B) with PCOS (n=5) calculated by GeNorm software. The selection of best stable genes based on the elimination of least stable genes with highest M values. This lead to identification of LPR10, ACTB and GAPDH genes as the most stable genes revealed low M value in both group (non-PCOS and PCOS). The program proceeds with stepwise exclusion of genes with relatively higher variable expression among the samples. The expression stability measure (M) is the average of the stability values of the remaining genes. The lower the M, the more stable the gene in the tissue. M is based on the expression ratio of two genes (pair comparison).

Determination of expression stability of selected reference genes by *NormFinder* analysis genes in two groups. Three genes were chosen based on stability ranking and minimal inter and intra group variation: GAPDH, LPR10, and ACTB, 0.0029, 0.0029 and 0.005 in non-PCOS **fig 3.11 A**; and ACTB, LPR10, and GAPDH, 0.113, 0.122, and 0.069 in women with PCOS **Fig 3.11 B**.

A



Gene Name SD Acc. SD

GAPDH 0.0029

LPR10 0.0029

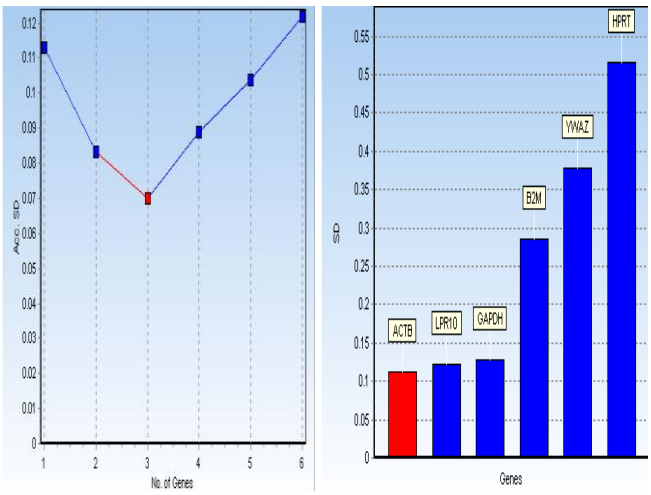
ACTB 0.005

B2M 0.1112

HPRT1 1.5958

YWAZ 1.919

B



Gene Name SDAcc. SD

ACTB 0.1131

LPR10 0.1223

GAPDH 0.1275

B2M 0.2863

YWAZ 0.3771

HPRT 0.516

Figure 3.11 shows determination of the most stable reference genes using Normfinder software in SAT of women (A) without PCOS (n=5) and (B) with PCOS (n=5). The main objective of the software program is to identify candidate reference genes with an inter group variation as close to zero as possible - and with small intra-group variation. In both groups, it was found that GAPDH, ACTB and LPR10 are the most stable genes.

3.1.2.4.2 Assay efficiencies detection

The optimisation and the performance of the assay were evaluated by detection of the linear regression line along with the co-efficient of determination R2. Linearity of the standard curve measures the variability among the assay replicate, and whether the amplification efficiency is the same for the different starting template copy. A perfect qRT–PCR is a reaction characterised by r >0.99 or R2 >0.98, **table 3.5**.

The other parameters used to detect the performance of the assay are the calculation of the assay efficiency (E). Efficiency of the assays means the ability of the reaction to double the amount of PCR product during each cycle (q-PCR guide Eurogentec). The best indicator for a reproducible assay is when the E value of the assay is close to 90-100%, **table 3.5**.

	Gene	Efficiency (%)	R_2
Reference genes	ACTB	106.02	0.979
	GAPDH	94.27	0.993
	LRP10	103	0.997
Target genes	CYP17A1	99.82	0.998
	AKR1C3	98.21	0.995

Table 3.5 Efficiencies of reference gene and target gene assays. R2 values determine a high goodness of fit of data to the trend line, which was used for the efficiency calculations.

3.1.2.5 Discussion

The analysis of gene expression by RT-PCR requires stringent normalisation in order to eliminate non-biological variables. Using reference genes is one of the most common strategies employed (Vandesompele, Kubista et al. 2009). However, choosing unsuitable housekeeping genes is one of the most common causes of erroneous results (Dheda, Huggett et al. 2005).

The method of choice for studying gene expression here is relative quantification. However, the accuracy and reliability of this method is based upon the choice of reference genes (Bustin 2000). Whereas the use of an internal control is needed to avoid and correct the experimental and non-biological variation, a non-ideal reference gene can lead to misinterpretation of data by introducing incorrect variation or masking the real variation. However, there is no one ideal reference gene which is suitable for all experiments. Therefore, most of the commonly-used endogenous controls have been documented to be sensitive to the experimental conditions and tissue under investigation (Thellin, Zorzi et al. 1999, Schmittgen and Zakrajsek 2000). Hence, it is recommended to validate reference genes before their use.

In view of this, multiple systems have been designed to determine the most stable genes (Vandesompele, De Preter et al. 2002). In this study, two of these commonly used approaches have been used to validate and compare a set of chosen reference genes, such that validated reference genes can be recognised and used in relative quantification research, including adipose tissue.

The ideal method would be to make a wide genome review of human adipose tissue in order to determine the potentially stable reference genes, before applying the validation process. Nevertheless, as a result of the complexity and expensive nature of this step, we limited ourselves to the optimisation of a group of endogenous controls, which are commonly used in studies involving adipose tissue. Although screening all tissues samples under all experimental conditions when selecting suitable reference genes is recommended, it is not always practically feasible. Hence, we advocate a monitoring protocol after initial screening of tissues, then re-screen when appropriate.

Firstly, we restate the significance of always standardising tissue samples at the RNA level, i.e. using the same concentration of RNA for all cDNA synthesis reactions. Indeed, differences in reverse transcription protocols can significantly affect downstream qRT-PCR assays (Bustin, Dhillon et al. 2015). Secondly, after choosing the reference genes - which are supposed to be stable in the tissues examined - any significant changes in Ct values must be monitored.

If Ct values change by more than 2 cycles between biological replicates, selection of other reference genes should be considered; after additional screening tests, if necessary.

Thirdly, the use of a geometric mean from at least three reference genes to normalise data has been recommended to adjust for the smaller changes in expression of the reference genes (Vandesompele et al. 2002). In this method, significant changes to the expression of reference genes following experimental conditions can be detected, eventually avoiding reporting of significantly inconsistent data.

Quite significantly, the investigation of the expression level of the reference genes themselves- especially for GAPDH and ACTB (Bustin 2000) - showed variation in different tissues and in the condition of the experiments. In adipose tissue, the variation in the expression of these reference genes was found to be based on the presence of obesity and DM. These observations necessitate the requirement to validate the reference genes for adipose tissue research (Catalan, Gomez-Ambrosi et al. 2007).

In this study, we used real-time PCR to identify genes with low variation in the expression level in the SAT of women, with and without PCOS. GAPDH, ACTB and LRP10 were identified as the genes with highest expression stability among the six selected genes.

The biological function of LPR10 is still incompletely known. The mouse homologue to LPR10, lrp10 (also known as lrp9) mediates cellular uptake and hydrolysis of cholesterol esters in apolipoprotein E-enriched very-low-density lipoprotein *in vitro* (Sugiyama, Kumagai et al. 2000).

GeNorm and Normfinder programs were used to determine the most suitable gene of a panel of six human genes, which are commonly used as reference genes in adipose tissue. In the GeNorm algorithm, the stability of the expression of a given gene (M) is calculated as the mean standard deviation of the log-transformed expression ratios among samples, relative to the other reference genes in the panel of genes. This is followed by exclusion of the gene with the highest M-value (i.e. the least stable gene) from the gene panel until the genes with the smallest M–value are obtained (i.e. the most stable genes) (Vandesompele, De Preter et al. 2002). In order to identify the most stable genes, Hellemens and his colleagues advise using a M≤0.5 (Hellemans, Mortier et al. 2007).

ACTB, GAPDH, and LPR10 were recognised as the most stable genes for non-PCOS; whereas LPR10, ACTB, and GAPDH were identified as the most stable genes in the PCOS group from chosen genes (YWAZ, HPRT1, B2M, ACTB, GAPDH, and LPR10). It is interesting to note that in each group LPR10, GAPDH, and ACTB were identified as the 3 most stable genes. (Ling and Salvaterra 2011) have observed that the stability of the candidate genes (with GeNorm analysis) are changed even when the samples had the similar tissue.

NormFinder suggests that GAPDH, ACTB, and LPR10 are relatively stable across the adipose tissue of both groups. Other genes were scored as borderline suitable, and included B2M, HPRT1, and YWAZ. GeNorm software suggested LPR10, ACTB, and GAPDH to be stable enough for normalisation in the non-PCOS and PCOS adipose tissues.

Similarity in stability rankings and outcomes between different analytical methods, such as GenNorm and NormFinder has been reported. The stability of genes was nearly the same using GeNorm and Normfinder. LPR10, ACTB, and GAPDH were identified as the best three genes. The human genes LPR10, GAPDH, and ACTB had the lowest standard deviation (SD) of Ct, an M value≤ 0.5 and thus the least variability across samples of both groups. These data indicate that these three genes are the best control candidates for this experimental study. The other 3 genes - HPRT1, YWAZ, and B2M gave the highest variance and are not recommended.

In conclusion, based on the evidence provided in the present study, we propose and recommend LPR10, ACTB, and GAPDH as stable and the most appropriate reference genes. These have not been previously identified for the purpose of expression studies of human adipose tissue.

3.1.3 Adipose tissue mRNA expression of steroid metabolic enzymes (CYP17A1, AKR1C3)

3.1.3.1 Introduction

Over the last few years, our understanding of physiology and pathophysiology of adipose tissue has been transformed. This includes understanding the complex role of adipose tissue in the regulation of several functions, such as reproductive and metabolic functions. In women, the main sources of androgens are the ovaries and adrenal glands that contribute equally to androgen levels (Barber and Franks 2013). In addition, up to 50% of circulating testosterone is derived from peripheral conversion of the weaker androgen, androstenedione and dehydroepiandrosterone sulphate (DHEAS) (Giagulli, Verdonck et al. 1989). In spite of this, the activity of several androgen metabolising enzymes in multiple peripheral tissues has been confirmed. The major peripheral site in which the activity of these enzymes occurred is adipose tissue (Barber and Franks 2013). In women with PCOS, the predominant source of high androgen secretion is likely to be ovarian and adrenal. However, it is important to appreciate that the androgen metabolising enzymes in the peripheral tissue may also determine the endocrine effect of the tissue.

The activity of steroidogenic processes in adipocytes is likely to have a critical role in the pathophysiology of PCOS, mainly hyperandrogenaemia, which is considered one of the main features of this syndrome.

This could explain the strong association between obesity and high androgen levels in women with PCOS.

In normal healthy female adolescents without PCOS during the pubertal transition period, enhancing steroidogenesis within adipocytes could explain the association between obesity and hyperandrogenaemia (McCartney, Prendergast et al. 2006).

A series of steroidogenic enzymes, including androgen metabolising enzymes, have been detected in human adipose tissue. Although the amount of sex steroid such as androgen or oestrogen is not excessive, dysregulation in the

conversion of androgen precursor to both active and inactive forms at the receptor level, is important in the pathogenicity of metabolic disturbance (Belanger, Luu-The et al. 2002). The study reported observed that a mutation in the P450 aromatase - which is responsible for the formation of oestrogen from androgen - may lie behind androgen excess and PCOS features in young women (Petry, Ong et al. 2005).

In PCOS patients, obesity and hyperandrogenism may occur as a result of high levels of 17-β HSD (which is responsible for the conversion of androstenedione to testosterone) and 5-α-reductase (responsible for the formation of DHT from testosterone) in adipose tissue (Barber, McCarthy et al. 2006). The expression of the main steroidogenic enzymes, including androgen metabolising enzymes, in adipose tissue of PCOS women has not been fully characterised.

In this study, we hypothesise that there is difference in the expression of the main steroidogenic enzymes CYP17A1 and the main androgenic metabolising enzyme AKR1C3 in SAT of women, with and without PCOS.

3.1.3.2 Objectives

In order to explore the role of adipose tissue in the pathophysiology of PCOS, the objectives of this study were:

- To study the expression level of the key enzyme in the steroidogenic pathway, CYP17A1 in SAT of women, with and without PCOS, within the reproductive age group.

- To investigate the expression level of the main androgen metabolising enzyme in steroidogenesis, AKR1C3, in SAT obtained from non-PCOS and PCOS women.

3.1.3.3 Methods

Total RNA was extracted from frozen SAT, obtained from women without PCOS (n=8) and with PCOS (n=8) at elective laparoscopic surgery employing the Trizol reagent method. Following reverse transcription of

mRNA, qRT-PCR was used to compare the mRNA expression of the two target genes (CYP17A1, and AKR1C3).

For normalisation, ACTB, GAPDH, and LPR10 were selected as reference genes after validation, **see section 2.6**. The Ct values were calculated and normalized using the GenEx software provided by the University of Nottingham (User Guide 1.0, MultiD analyses). The results from PCOS and healthy volunteers were analysed using GraphPad Prism version 6.0f, **see section 2.14** for statistical analysis of RT-PCR data.

3.1.3.4 Results

3.1.3.4.1 The relative mRNA expression of CYP17A1

We first attempted to determine the expression pattern of CYP17A1 mRNA in the SAT of women without PCOS and from women with PCOS, using the qRT-PCR technique. As shown in **fig 3.12**, CYP17A1 was detected in adipose tissue of both study groups. However, there was no significant difference (P >0.05) in the CYP17A1 mRNA expression level from adipose tissue of women with PCOS compared to non-PCOS women.

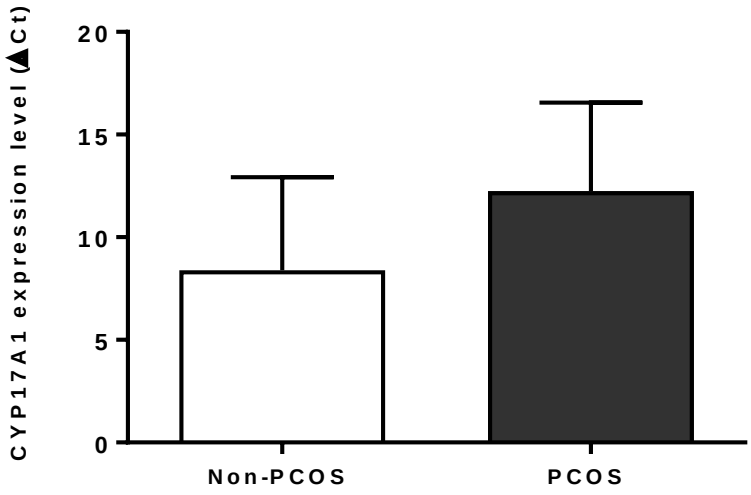


Figure 3.12 Relative mRNA expression of CYP17A1 in SAT of non-PCOS and PCOS women. The expression level of CYP17A1 mRNA was measured in adipose tissue of non-PCOS (n=8) and PCOS women (n=8) using the qRT-PCR technique. ACTB, GAPDG, and LPR10 were the references genes used to normalise gene analysis. There is no significant difference in the mRNA of CYP17A1, P >0.05. Data expressed as mean± SEM. Normalization includes reporting the ratios of the mRNA concentrations of the CYP17A1 gene to those of the reference genes' (ACTB, GAPDG, and LPR10) ΔCt. Quantitative RT-PCR was performed to determine the expression of CYP17A1 and a panel of choosing reference genes, data were analysed with GenEx and compared using ΔCt method.

3.1.3.4.2 The relative mRNA Expression of AKR1C3

Next we compared the expression levels of AKR1C3 between PCOS (n=8) and non-PCOS (n=8) women. The mean± SEM relative expression level of AKR1C3 mRNA in PCOS was 15.1±2.0, which was significantly greater (P=.0003) than that (3.3 ±1.1) of the control samples or non-PCOS. **Fig 3.10** shows the difference in the AKR1C3 mRNA expression in SAT of PCOS and non-PCOS women.

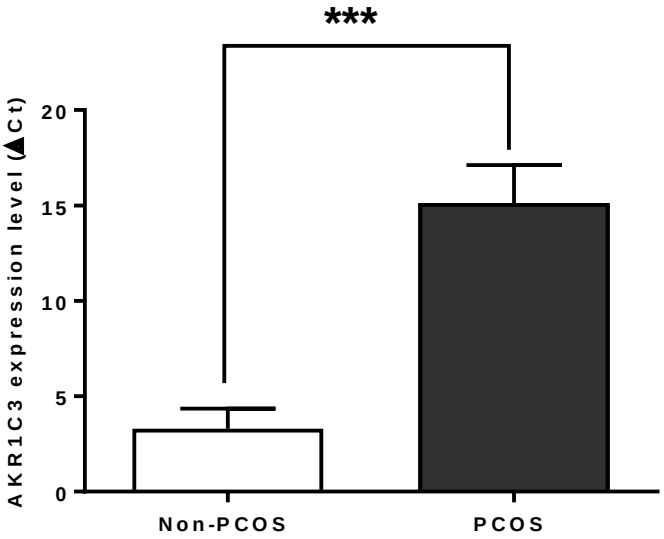


Figure 3.13 AKR1C3 was differently expressed in the SAT of PCOS (n=8) and non-PCOS (n=8). The expression level of mRNA of AKR1C3 was measured by the qRT-PCR technique using RNA extracted from frozen adipose tissue samples of two groups using Trizol reagent. The c DNA made after that was processed for PCR, and normalised to the following reference genes GABDH, ACTB, and LPR10. There were significant differences in the normalised AKR1C3 expression level P <0.05. Data is presented as mean± SEM, ** P <0.01.

3.2 Adipose tissue protein (AKR1C3) expression

The previous results provided impetus and attention to the study of the androgen metabolising enzyme, AKR1C3 protein, in adipose tissue of women with PCOS.

mRNA concentration is taken as an alternate for the corresponding protein concentrations; however, for most genes, the actual cellular player is protein (Nie, Wu et al. 2006). It has been determined that protein concentrations correlate with mRNA concentrations by only 20%–40% (Nie, Wu et al. 2006). Until now, it has been difficult to precisely detect protein concentrations compared to mRNA concentrations. As a result of detection of numerous post-transcriptional regulation mechanisms, mRNA levels only partly correlate with protein concentrations (Brockmann, Beyer et al. 2007). Better interpretation of results obtained from mRNA measurements in studying protein expression and understanding translational regulation is needed (Kolkman, Daran-Lapujade et al. 2006).

The aim of this study was to investigate the expression of AKR1C3 *in vivo*, in non-PCOS and PCOS; and to describe the relationship between AKR1C3 and adipose tissue in PCOS using WB.

3.2.1 Objectives

- To study and compare the expression of AKR1C3 in non-PCOS versus PCOS adipose tissue.

3.2.2 Methods

AKR1C3 protein expression was detected and evaluated by WB technique (which has been described in **see section 2.9**) in non-PCOS (n=3) adipose tissue samples and PCOS (n=3). The basic clinical patient's data **see section 2.1, tab 2.1.**

3.2.3 Results

3.2.3.1 Adipose tissue target protein (AKR1C3) expression levels

Non-PCOS lysates from adipose tissue were examined using the WB technique for expression of AKR1C3 (non-PCOS n=3 *vs*. PCOS n=3). **Fig 3.14** shows expression at 37KDa was seen in adipose tissues taken from both group's lysate, suggestive of the presence of this enzyme. Also, it shows that AKR1C3 was expressed less in PCOS compared with non-PCOS human adipose tissue. All lanes were normalised against β-Actin.

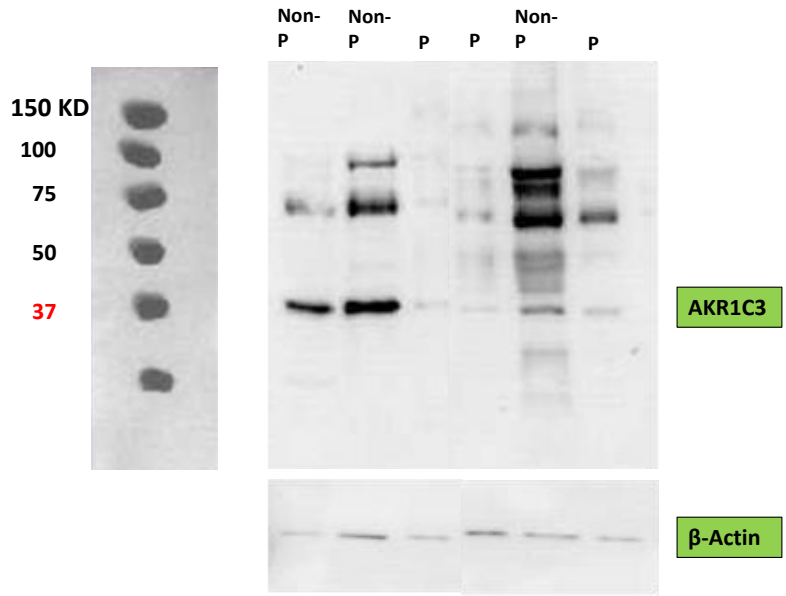


Figure3.14 Western immunoblot analysis of AKR1C3 protein expression in non-PCOS (n=3) and PCOS (n=3) frozen SAT. Total proteins were extracted from frozen adipose tissue samples of women without PCOS and with PCOS. The extracted proteins were electrophoresed on 12% of SDS-PAGE, and transferred to a nitrocellulose filter. Immune detection of AKR1C3 protein was performed by incubating with rabbit anti-human AKR1C3 polyclonal Ab at a 1:1000 dilution overnight and with goat anti-rabbit IgG secondary antibodies at a dilution of 1:30000. Each lane was loaded with 100µg of protein. Molecular weight markers were used to facilitate protein size detection. The position of the molecular weight marker is shown. The blot was then stripped and re-probed using β-Actin to control for loading of the protein. All blots were probed against placental controls (not shown). Protein was quantified by densitometry and the relative expression levels adjusted for B-actin.

Fig3.15 shows there is variation in band intensity between non-PCOS and PCOS adipose tissue lysate which was quantified by densitometry. This would suggest differences in AKR1C3 expression across the two groups. However, based on band intensity, there was no significant variation ($p>0.05$) between both groups. Densitometry for the bands was measured using specific software provided by Nottingham University, and normalized to reference protein β-actin; data were compared using t-test.

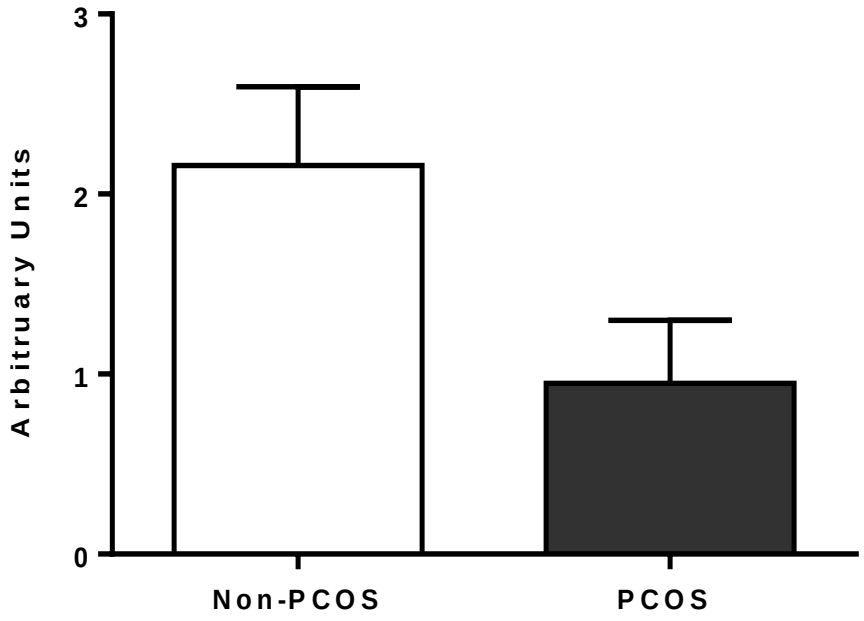


Figure 3.15 demonstrates the relative intensity of AKR1C3 protein expression in non-PCOS (n=3) and PCOS (n=3) SAT. Using β-Actin normalisation to measure the relative levels of band intensity in WB of AKR1C3 expression protein. The result shows there was no significant difference in the protein expression between non-PCOS and PCOS, P> 0.05 (=0.34). The result is mean± SEM. This data represents three experiments. The experimental procedures were as described in the text.

3.3 Discussion

In the present study, we investigated the expression level of two major enzymes in steroid synthesis, metabolism and production; namely CYP17A1 and AKR1C3 in SAT of women at reproductive age with and without PCOS, after matching for age and BMI. AKR1C3 gene represents 17β-HSD type 5, which forms testosterone from a precursor, androstenedione and DHEAS (Miller and Auchus 2011).

The results of this study detected the expression of two major enzymes (CYP17A1, AKR1C3) in SAT, but did not show any significant difference in the expression of CYP17A1 between PCOS and non-PCOS women. On the other hand, there was significant difference in the expression level of AKR1C3 (17β-HSD 5) between the two groups; quantitative analysis revealed a five-fold higher expression of AKR1C3 mRNA levels than in non-PCOS. Accordingly, the rate of conversion from androstenedione to testosterone speculates to be higher in adipose tissue of women with PCOS, and may be a mechanism whereby hyperandrogenism may ensue. Therefore, investigate the activity of AKR1C3 in women with PCOS could be considered one of the work considered in future.

These findings indicate that adipose tissue could produce its own steroid hormones, including androgens, and is consistent with the conclusion of Puch in 2002. In addition, recently this conclusion is supported by detection of CYP17A1 protein expression in adipocytes isolated from SAT of women (Cadagan, Khan et al. 2014). In their study, western blot technique was used to study CP17A1 expression; in our study we investigated at the mRNA expression level.

Based on the result of this study, it is suggested that local production of testosterone is high in SAT of PCOS, and it appears that androgen synthesis may depend mainly on the conversion of weaker androgens (especially from androstenedione) to testosterone instead of cholesterol. Therefore, SAT may be an important steroid inactivating - and androgen producing - tissue, and may have a significant role in androgen production in PCOS women.

AKR1C3 is reported to have a wide tissue distribution, and is suggested to have a significant role in androgen synthesis in peripheral tissue (Dufort, Rheault et al. 1999); (Dufort, Labrie et al. 2001). In 2004, Quinkler et al. found that 17β-HSD type 5 is the main active isoform in human adipose tissue. In the same study, they observed that the activity of this steroidogenic enzyme was enhanced in in vitro SAT taken from women with obesity (Quinkler, Sinha et al. 2004).

Accordingly, dysregulation in androgen metabolising enzymes in adipose tissue may have a key role in the development of PCOS, particularly hyperandrogenism. In addition, it may be one explanation for the higher androgenicity associated with PCOS. In addition, dysregulation in the metabolism of sex steroid in fat tissue may have a critical role in the determination of the gynoid vs. android pattern of body fat; increased synthesis of androgen by AKR1C3 was directly associated with obesity in the women (Kuchenbecker, Groen et al. 2010).

Furthermore, accumulation of fat - particularly abdominal and trunk - is associated with disturbances in ovulation, which is one of the major features of this syndrome (Kuchenbecker, Groen et al. 2010).

Moreover, in 2012, Wang et al. found that in adipose tissue of PCOS, 3β-HSD enzymes were significantly more highly expressed in PCOS patients when compared with non-PCOS patients. This enzymatic system efficiently converts pregnenolone to progesterone, and dehydroepiandrosterone to androstenedione. This means a high level of precursors are provided to form androgen by the action of enhanced AKR1C3 (Wang, Li et al. 2012). This is in association with a decrease in the local production of DHT, oestrone, and oestradiol. This is due to low expression in 5α-reductase, which is responsible for the conversion of testosterone to DHT; and P450 aromatase, which forms oestradiol/oestrone from testosterone in adipose tissue of women with PCOS, further supporting this contention (Wang, Li et al. 2012)

Moreover, the total amount of steroid in the adipose tissue was observed to be 40-400 times more than plasma (Deslypere, Verdonck et al. 1985). In addition, a positive gradient of steroid between tissue and plasma has been

described (Szymczak, Milewicz et al. 1998). Therefore, in PCOS, abnormal metabolism of steroid hormones and androgen in adipose tissue, may contribute to the serum levels of androgens. It could also exert a critical local biological effect, for instance, on insulin resistance and adipokine production (Wang, Li et al. 2012).

These results suggest that local synthesis of testosterone increases in SAT of women with PCOS. In addition, it suggests that the regulation factor/or factors, which regulate or modify the expression of CYP17A1 and AKR1C3 in PCOS is/or are different.

The results of this study might suggest the presence of steroidogenic pathways in SAT. In PCOS, our data revealed that SAT expresses significant levels of androgen metabolising enzyme. There was an increase in the expression of testosterone synthesis enzyme. Therefore, SAT may depend more on the conversion of androgen precursor, androstenedione in testosterone production - instead of cholesterol. Therefore, there is a possibility of the contribution of adipose tissue to the development of the hyperandrogenic state in women with PCOS, and might better explain the association between obesity and anovulation in this syndrome. As this is just mRNA results, it is important to support these findings by other laboratory methods such as Western blot - which will be discussed in the following section; and also have to supported by measuring the rate of AKR1C3 enzyme activity in adipose tissue of women with PCOS and compare to that in normal women, this was not studied, hence can be considered as one of the limitation of this study.

With regard to AKR1C3 protein expression, our study on steroidogenic enzymes in adipose tissue has shown that mRNA of AKR1C3 catalysing androgen formation is upregulated in PCOS adipose tissue. Here I evaluated the AKR1C3 expression by WB in non-PCOS and PCOS women.

The results of this study have shown that it is easy to detect AKR1C3 mRNA and protein by PCR and WB. However, there is inconsistency between AKR1C3 mRNA levels and protein expression in PCOS adipose tissue.

Studying non-PCOS and PCOS human adipose tissue for AKR1C3 expression, identified multiple bands. Based on the fact that AKR1C3 has multiple isoforms (in humans there are 8 isoforms) (Byrns, Jin et al. 2011)).

In order to optimize the technique to avoid the appearance of these bands, we found difficulties, such as did not detect of target bands. Also, degradation of the lysates from the freeze thawing process occurred, and when this happened new samples were required. With limitation of samples and time, it is difficult to process enough numbers of samples and repeat the experiments. However, with time limitations and with limitations in the availability of PCOS biopsies, we could not repeat the experiments. For the purpose of comparing AKR1C3 enzyme expression between groups, this result was appropriate, but may be considered in future work.

Although of that there were some limitations in performing this experiments some of these technical related to the WB optimization, other related to the nature of studying protein, however we may suggest that based on these primary results, the expression levels of AKR1C3 protein were not significantly lower than that in the adipose tissue of PCOS women when compared with non-PCOS.

Our suggestion on the variation in the expression level of AKR1C3 protein between non-PCOS and PCOS tissue samples may be related to altered metabolic states of the tissue, which may impact on the protein expression. Studies on the characterisation of AKR1C3 expression in adipose tissue of obese subjects with and without metabolic complications reported that AKR1C3 expression levels were reduced during and after diet-induced weight-loss - in

comparison with its level before the start of the diet (Svensson, Gabrielsson et al. 2008). Also, Cho investigated the expression of AKR1C3 in normal skin tissue and diabetic skin tissue. He found that AKR1C3 was expressed less in diabetic skin tissue (Cho 2013).

Furthermore, in order to suggest the variation in the AKR1C3 protein expression, it is important to consider the factors, which affect the correlation between expression of this enzyme at gene and protein expression level.

One of the key post-translation factors affecting mRNA-protein correlation is the half-life of protein, which depends on multiple factors: stability of protein, post-translational processing such as phosphorylation, and glycosylation. Half-lives of proteins are highly variable, ranging from a few seconds to several days (Maier, Güell et al.).

As mentioned above in the Introduction chapter, there are multiple enzymes that are currently known to have the capability to metabolise the conversion of androstenedione to testosterone, particularly AKR1C3, HSD17B1, HSD17B3, AKR1C1, and AKR1C4 (Dufort, Labrie et al. 2001); (Dufort, Labrie et al. 2001, Labrie, Luu-The et al. 2005). AKR1C3 is expressed by adipose tissue, giving this tissue the ability to be involved in testosterone synthesis (Penning, Burczynski et al. 2001). However, studies on the protein's expression in adipose tissue of women with PCOS compared to non-PCOS women is limited - this may be the first study in this subject.

In steroidogenic tissues, the main function of 17β-HSD (AKR1C3) is the conversion of androstenedione to testosterone. However, in *Escherichia coli*, this conversion is achieved at an inefficient rate (k_{cat}/K_m of 4.54 min/mM substrate) (Penning, Burczynski et al. 2001). Therefore, it is unclear whether this is the primary enzyme responsible for this reaction in the ovary (Goodarzi, Jones et al. 2008).

Quinkler and his colleagues reported that AKR1C3 protein is determined in the human adipose tissue using WB analysis (Quinkler, Sinha et al. 2004). To our knowledge, there are no previous studies regarding AKR1C3 in adipose tissue of PCOS women. Although our results are resonant due to some limitations as mentioned above, but are interesting as they show that AKR1C3

mRNA and protein are inconsistently expressed in adipose tissue, PCOS adipose tissue group. It was determined that AKR1C3 is a potential candidate gene in the development of hyperandrogenemia in women with PCOS due to its role in the production of androgen in the ovary (Quinkler, Bujalska et al. 2006). It was demonstrated that polymorphisms in the AKR1C3 genes are not associated with serum testosterone levels in women (Goodarzi, Jones et al. 2008). Hence, currently, it appears that the genetic component does not link

excess testosterone to AKR1C3. However, it may be postulated that human AKR1C3 may well afford a mechanism for adipose tissue to be involved in the circulating pool of testosterone in women with PCOS (Goodarzi, Jones et al. 2008).

Even though it is true that AKR1C3 enzyme is important for testosterone production in human tissue, further studies are needed to detect whether expression of small amounts of this enzyme in adipose tissue of PCOS could contribute to high testosterone production in PCOS. This could be performed by repeating the experiment with a large number of samples, and by using other techniques such as immunofluorescence staining experiments, and by measuring the activity of this enzyme in adipose tissue of women with PCOS.

We regretted not to determine the protein level by using other techniques such as immunofluorescence and enzyme activities due to limited sample volume, and time. More attention should be given to sex steroid inactivation in adipose tissue. The number of samples in this experiment was relatively small and the results will therefore need to be verified by other larger studies.2

3.4 Impact of hormones (insulin± LH) on androgen metabolism in adipocytes

3.4.1 Optimisation of primary cell culture system

3.4.1.1 Introduction

With the identification of adipose tissue products (Ahima and Flier), and the determination of the expression of adipocytes for different receptors which mediate the response of adipocytes to several hormones such as insulin and androgen (Flier 1997) -adipose tissue is considered an active endocrine organ. The main cellular component of adipose tissue is the adipocyte (Kim and Kim 2003). The investigation of adipocyte function and dysfunction has become an important subject since the detection and association of obesity with other abnormalities, such as insulin resistance (Guilherme, Virbasius et al. 2008).

The ability of mature adipocytes to proliferate is low and there is controversy about the presence of this capability (Rodriguez-Cuenca, Monjo et al. 2005).

Therefore, the best alternative to this is *in vitro* differentiated adipocytes to establish high yields through culturing and maintenance of pre-adipocytes to adipocytes, through differentiation treatments.

Differentiation or 'adipogenesis' is a process through which premature adipocytes adopt the phenotypical, biochemical, and functional properties of terminally differentiated cells (Yang and aiping 2008).

In order to understand the cellular and molecular basis of adipose tissue in non-PCOS and PCOS, it is apparent that it is critical to establish a primary adipocyte monolayer cell culture system to perform these studies.

The main aim of this aspect of the study was to establish a primary cell culture system that could be used for investigation of the impact of hormones (insulin± LH) on androgen metabolism in adipocytes of PCOS women, in order to understand the relationship between these hormones and hyperandrogenism in PCOS.

3.4.1.2 Objectives

- To establish primary human adipocyte cultures from SAT biopsies and achieve differentiation of pre-adipocyte culture, to be ready for application of treatments and for PCR and ELISA assays.

3.4.1.3 Methods

3.4.1.3.1 3T3-L1 cell line culture and differentiation

Although 3T3-L1 cell line is not one of the main targets of this study, it was used at the start to transfer the protocol applied in primary cell culture. Cultures of the 3T3-L1 cell line at passage 6 were taken from liquid nitrogen storage, cultured and maintained until confluence. Then pre-adipocytes were transferred to T75 flasks to expand the cells. When they reached 80-90% confluence, pre-adipocytes were then plated into 24 well plates at a density of $3x10^5$ cells per well - and grown to 80-90% for differentiation in the incubator. After successful culture and *in vitro* differentiation, the protocol was applied to target cells (primary cell culture).

3.4.1.3.2 Primary pre-adipocyte culture and differentiation

(**See section 2.11 for details)**

3.4.1.3.3 RNA extraction from *in vitro* differentiated adipocytes

The method of RNA extraction is the same as that used to isolate RNA from frozen adipose tissue samples. The difference and steps are detailed (**see section 2. 2.1.2**).

3.4.1.3.4 Collection of culture media from prepared cell culture

After preparation of the cell culture and treatment, the supernatant was collected and stored in the -80°C freezer to be used for ELISA assay (**see section 2.13.1**).

3.4.1.4 Results

3.4.1.4.1 Culture, differentiation and assessment of the 3T3-L1 cell line

Fig 3.16 shows 3T3-L1 cell morphology. The cells are small and spindle-shaped with low confluence, grown in maintenance media.

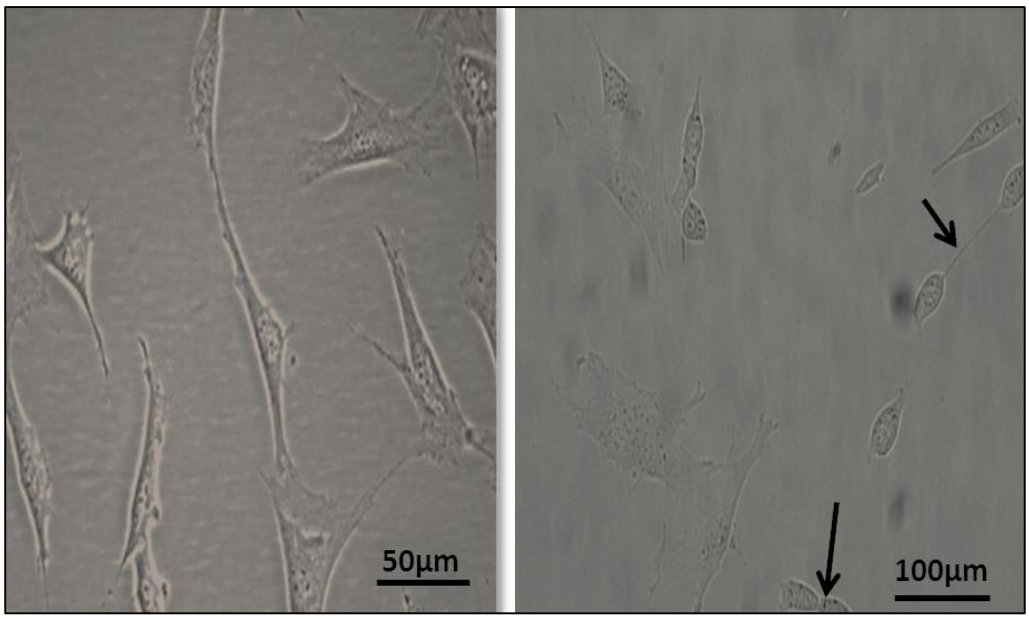


Figure 3.16 A photomicrograph of 3T3-L1 fibroblast-like pre-adipocytes cell line. The monolayer cells showed typical fibroblast morphology, flat and beginning to form a tight mosaic formation; arrows refer to the cell division events observed during culture.

With growth of the 3T3-L1 cells, confluence was attained in 4 to 6 days**, (fig 3.17 a, b, c)**. At this time, a few of the cells showed small lipid droplets in their cytoplasm. This observation highlights the adipogenic potential of this cell line (Green and Kehinde 1975). The cells are tightly packed together, enabling them to support each other. At this stage, cells become ready to initiate differentiation in response to the addition of induction differentiation media (IDM).

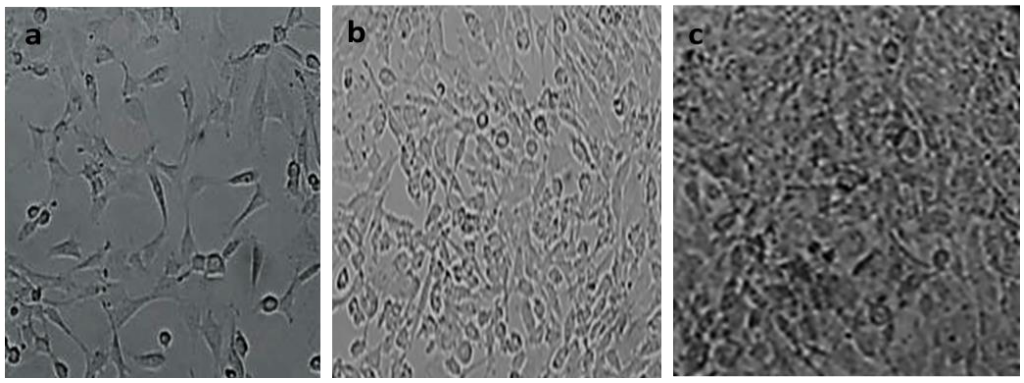


Figure 3.17 Variation in the rate of confluence of 3T3-L1 cells during growth. (a) Growth of 3T3 L1 cells at passage 12 on day 1. The adherent cells appeared small, with spindle shaped morphology and low confluence. (b) On day 3, the adherent cells became sub-confluent and formed a monolayer of cells which did not cover the entire plate surface. (c) On day 5, the cells reached 90% confluence, and covered all the plate surface with a round shape cell appearance (magnification for all figures is ×100).

The IDM was added when confluence was 80-90% (**Fig 3.18**), when the cells contact each other. At this point, the growth of pre-adipocytes is arrested because of their increasing number and due to the secretion of and exposure to initiation factors (CCAAT/enhancer binding protein a (C/EBP-a and PPAR-Y) from adjacent cells. This enhances the differentiation process (Gregoire, Smas et al. 1998). After induction of differentiation, small lipid droplets were observed under the microscope in about 70% of the cells. Droplets are observed as a circular spot inside cells, as the cell shape becomes round. With differentiation, the size of the cells increases due to lipid formation - as a result the cells detach easily. At this stage, careful handling during media changes was required. In addition, as a result of increased lipid in the cells, sheets of the cells tearing away from the base of the well were observed. Every day observation of the cells was needed in order to decrease this loss.

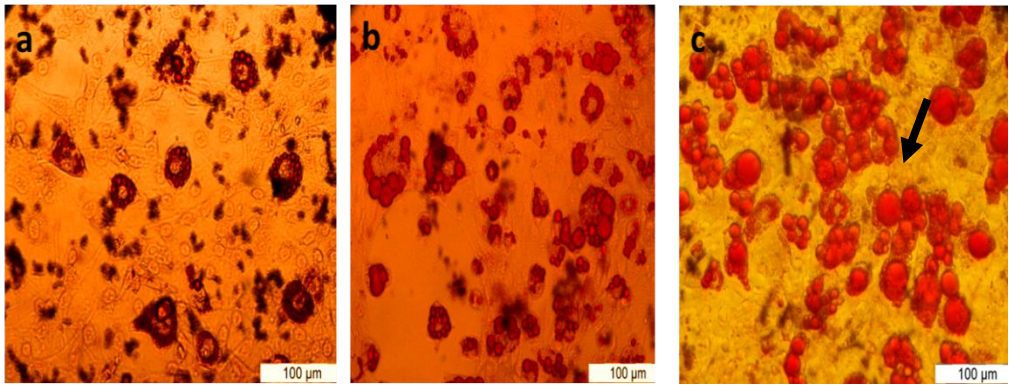


Figure 3.18 demonstrates the changes in the number and size of lipid droplets in the cytoplasm of differentiated 3T3-L1 adipocytes during days of differentiation. These increased through days of differentiation, and the difference is quite clear, on (a) day 8, (b) day 14, and (c) day 21 of differentiation. The accumulation of lipid droplets in the cytoplasm had been evaluated by Oil Red O stain on day 21. Oil Red O staining was used to confirm *in vitro* differentiation of adipocytes, and to assess the increased lipid content of adipocytes compared to undifferentiated cells. On day 21, the cells were stained with Oil Red O, (b) which stains lipid vacuoles, enabling visual quantification of lipid content in cells by microscopy. The black arrow refers to the cytoplasm of the cells filling with lipid droplets.

The program of adipocyte differentiation includes several distinct phases (Cornelius 1994). Pre-adipocytes enter a temporary quiescent state arresting at the G_0/G_1 cell cycle boundary, when they reach confluence (Scott 1982). At confluence, the arrest of growth seems to be a prerequisite for subsequent differentiation. However, arrest of this cell cycle is overcome by mitotic and adipogenic inducers. 3T3-L1 confluent pre-adipocytes are characteristic in that serum alone is incapable of inducing re-entry of contact-inhibited pre-adipocytes into the cycle of cell. To induce re-entry of density-dependent, growth-arrested pre-adipocytes into the cell cycle, specific adipogenic agents are required. Addition of IDM (induction differentiation media), consisting of isobutylmethylxanthine (a cAMP phosphodiesterase inhibitor), dexamethasone (D), insulin, and foetal bovine serum (FBS) (MacDougald 1995). Mitotic clonal expansion is defined as the cells re-entering the cell cycle and undergoing multiple rounds of mitosis (Student 1980). The initiation of the clonal expansion phase includes the expression of "immediate early" genes, C*/EBPβ,* and *C/EBPδ*, to direct pre-adipocyte confluence from G_0 into G_1. These genes are expressed during the first few hours after induction of differentiation. At mitosis, DNA replication is suggested to change the accessibility of promoter/enhancer elements to agents needed for the transcription of genes included in the beginning of differentiation (Cornelius 1994). This is followed by the expression of transcription factors, C/EBPα and PPARγ, which stop mitotic clonal expansion and co-ordinately activate transcription of adipocyte genes (Lin 1994). Mitotic clonal expansion of growth-arrested pre-adipocytes seems to be required for optimal differentiation. Therefore, introduction of sub-confluent proliferating pre-adipocytes to differentiation inducer leads to poor differentiation (Student 1980)

In addition to culture, the rate of differentiation was assessed. **Fig 3.19** shows the significant variation (P <0.05) in the rate of differentiation during the period of differentiation. This demonstrates that using induction and maintenance differentiation media**,** the rate of differentiation was significantly higher in comparison with cells growing in media without induction differentiation factors.

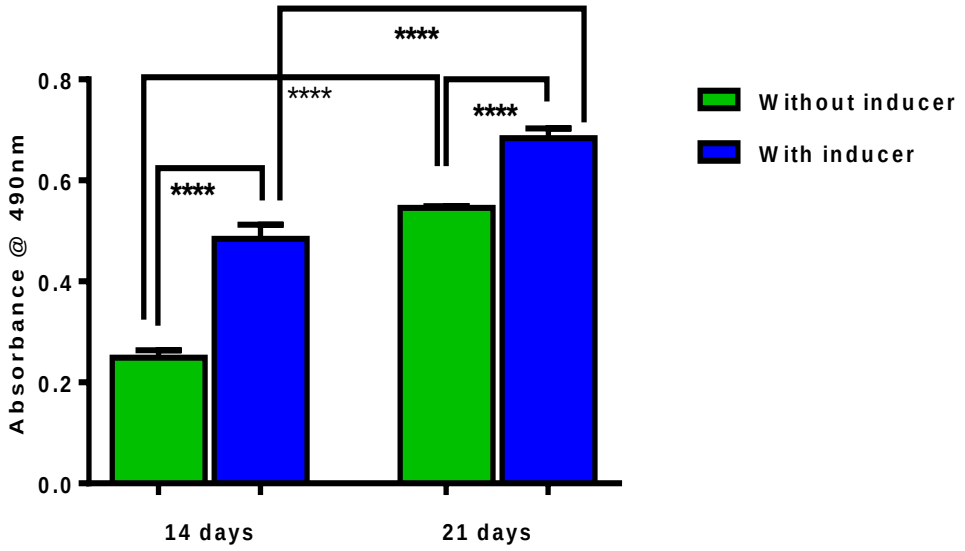


Figure 3.19 shows the differences in the rate of lipid accumulation in the cytoplasm of differentiated 3T3-L1 pre-adipocyte cell lines - with and without inducer (hormones) among days of differentiation. The 3T3-L1 cells were incubated in DMEM without inducer supplemented with foetal calf serum; this was considered a control (differentiation not initiated), whilst other cells were cultured in DMEM containing Insulin, IBMX and dexamethasone (inducer). Both cell cultures were stained with Oil Red O stain and the dye was extracted using 4% Igebal CA360. Extracted dye was measured in a plate reader at 490nm on day 14 and 21. Data are expressed as means ±SEM. ****P value <0.0001.

3.4.1.4.2 Culture of human adipocytes from women with and without PCOS

3.4.1.4.2.1 Demography of enrolled women.

For detail see **section 2.1.2, table 2.1.** In this experiment, PCOS (n=5) patients were compared with age and BMI –matched non-PCOS (n=5) women. For biochemical variation, based on the recent studies, which have demonstrated that DHEAS is less reliable than testosterone with only 5% of patients with hirsutism examined showed high level of DHEAS (Yamamoto et al., 2002). There is a significant increase in testosterone level (>2.8nm/l) and LH of women with PCOS.

3.4.1.4.3 Isolation, culture and differentiation of adipocytes of non-PCOS and PCOS women

After isolation from adipose tissue, pre-adipocytes were established *in vitro*, taking around 2-4 weeks to reach confluence in T25 flasks before trypsinisation to a T75 flask for expansion (**fig 3.20**). Morphologically, the cells were elongated and spindle shape growing to 90-100% confluence and forming a tight cellular matrix, which enabled adherence to the flask base**.** Therefore, as suspected by (Quinkler, Sinha et al. 2004) there was no need to coat the flask surface with fibronectin. As with the 3T3-L1 cell line, regular maintenance and observation was required with media change every two days.



Day 1 Day 4 Day 8 Day 14

Figure 3.19 Photomicrographs at ×200 magnification of human pre-adipocytes during its growth - after isolation from fresh adipose tissue samples by enzyme digestion. At day 1 morphologically the cells are flat, spindle shaped cells, and have similar appearance in culture to fibroblasts or smooth muscle cells; day 8 shows the cells increasing in number; and by day 14 the cells are confluent (80-90%).

3.4.1.4.4 *In vitro* differentiation of adipocytes

Figure 3.20 shows mature adipocytes staining with ORO staining.

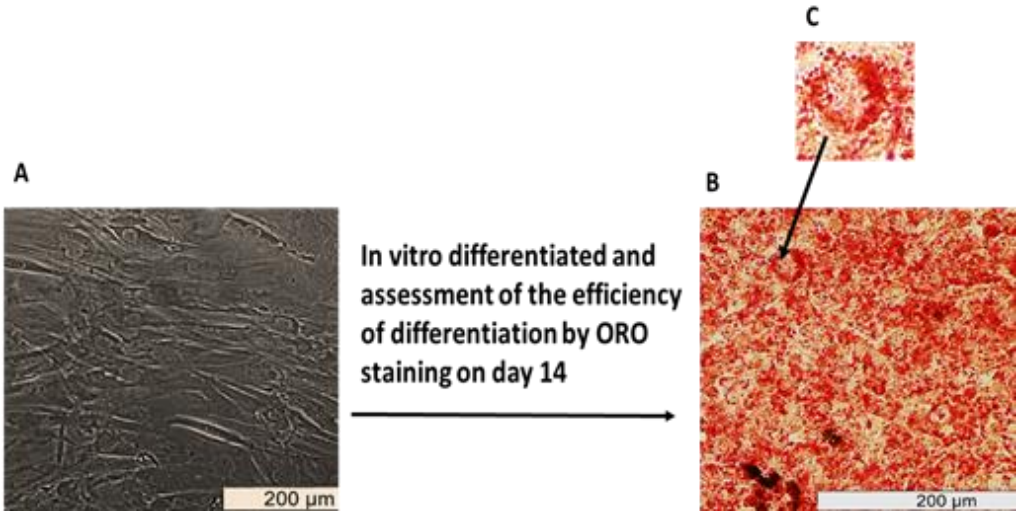


Figure 3.20 Human adipocytes (non-PCOS), *in vitro* differentiated over 14 days, and the adipogenic differentiation was evaluated using Oil-Red O staining. A: demonstrates undifferentiated confluent pre-adipocytes ready to start induction of differentiation. B: accumulation of lipid drops visible in differentiated cells; these stain red after Oil Red O staining, distributed in the cytoplasm of mature or differentiated adipocytes. C: At this time, the cells appear rounded, and are considered mature adipocytes, suitable for most assays or experiments.

3.4.1.4.5 Evaluation of *in vitro* differentiation of adipocytes

To validate maturation of adipocytes, Oil Red O staining was used. **Fig 3.21** shows large and small lipid droplets visible with staining with Oil Red O. We are able to measure differentiation using Oil Red O staining over a 3-week period. Removal of the Oil Red O was followed by absorbance measurements. There is a significant difference (P <0.0001) in the differentiation between non-PCOS (n=5) and PCOS (n=5) adipocytes **tab 3.5, fig. 3.22**.

	Mean± SEM
Non-PCOS(n=5)	0.72±0.03
PCOS(n=5)	0.38±0.05

Table 3.5 shows the difference in the rate of differentiation of adipocytes of non-PCOS and PCOS. Data presented as mean ±SEM. ****P<0.05

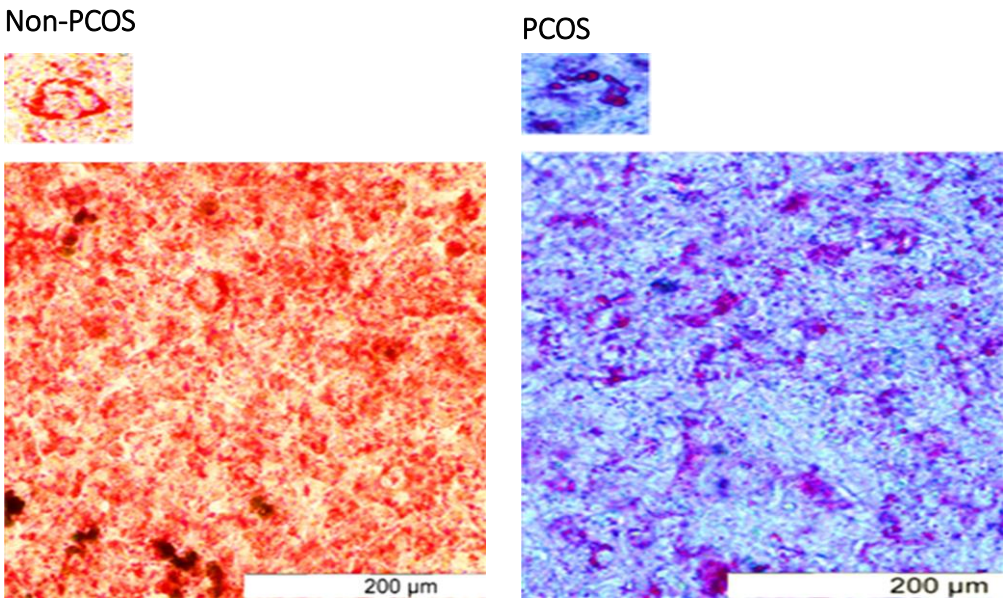


Figure 3.4 shows 2 week differentiation Oil Red O Staining of non-PCOS and PCOS adipocytes. 80% of the cultures have matured with large lipid vacuole formation observed by Oil Red staining.

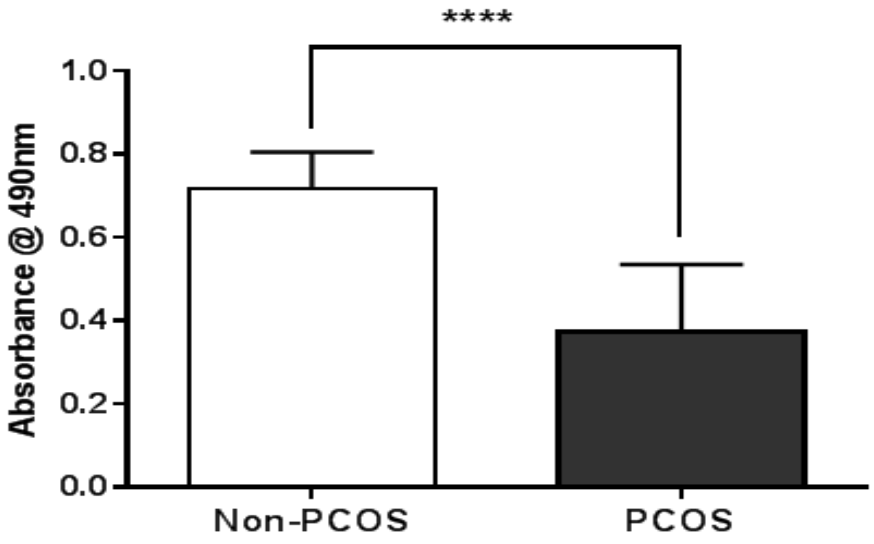


Figure 3.22 Non-PCOS and PCOS differentiation adipocytes (n=5 vs.5). After *in vitro* differentiation of human adipocytes, the media was removed at 2 weeks' differentiation and the cells stained with Oil Red O. Then the Oil Red O stain was extracted and its absorbance at 490nm measured by spectrophotometer. Data presented as mean ± SEM. **** P <0.0001.

3.4.1.4.6 Assessment of isolated RNA quality

Fig 3.23 shows that there is a significant difference in the concentration of extracted RNA between pre-adipocytes and mature adipocytes (P =0.02). However, there is no difference in the purity of extracted RNA between cells before and after differentiation P >0.05.

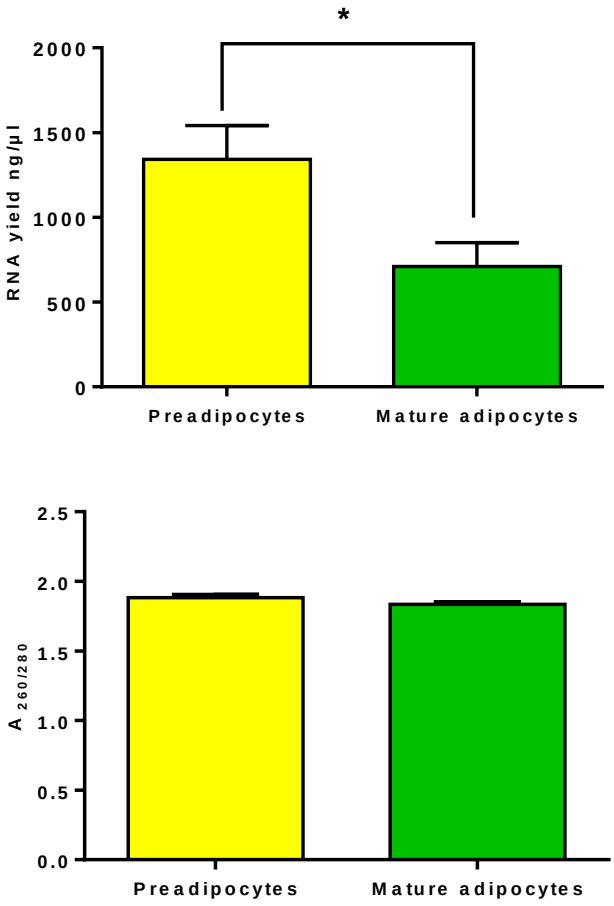


Figure 3.24 Isolation of total RNA from SAT. Total RNA was extracted from human adipocytes before differentiation (pre-adipocytes) and after differentiation (mature adipocyte) by Trizol reagent method; and assessed in term of quantity and purity by NanDrop spectrophotometer. Data presented as mean ± SEM.* P <0.05.

The integrity of extracted RNA from cell culture monolayer cells was evaluated by gel electrophoresis.

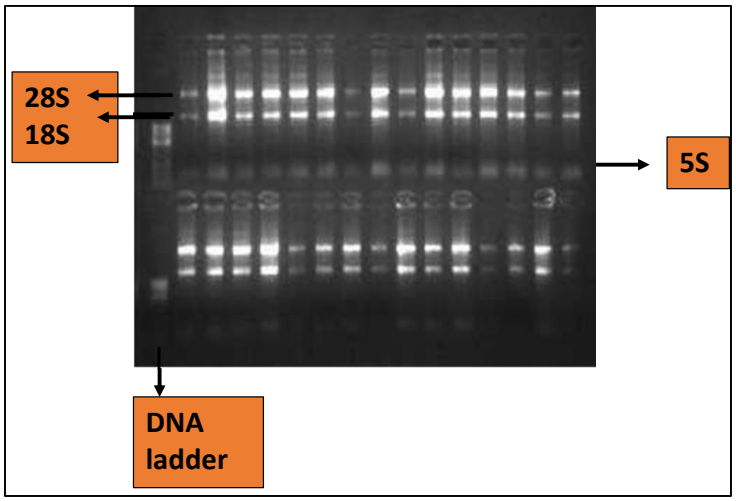


Figure 3.25 Gel electrophoresis of RNA isolated from *in vitro* differentiated adipocytes extracted from human adipose tissue. It shows the ultraviolet (UV) exposure of the intact total RNA on the 1% agarose gel solution of adipocytes; "L" stands for 1kb DNA ladder. The 18S, 28S and 5S bands are seen, the 28S band being twice as intense compared to the 18S.

3.4.1.4.7 Comparison of RNA extracted from different sources

In order to explore how the source of RNA affected the quality of RNA extracted, we compared the quality of RNA extracted in terms of its quantity and purity from adipose tissue (AT) and human primary cells (pre-adipocytes, M mature adipocytes). **Fig 3.26** shows the result in terms of concentrations obtained from human pre-adipocytes. The concentration of total RNA extracted from pre-adipocytes is significantly greater (P=0.0002) than that obtained from adipose tissue. The lowest concentration of total RNA was obtained from adipose tissue.

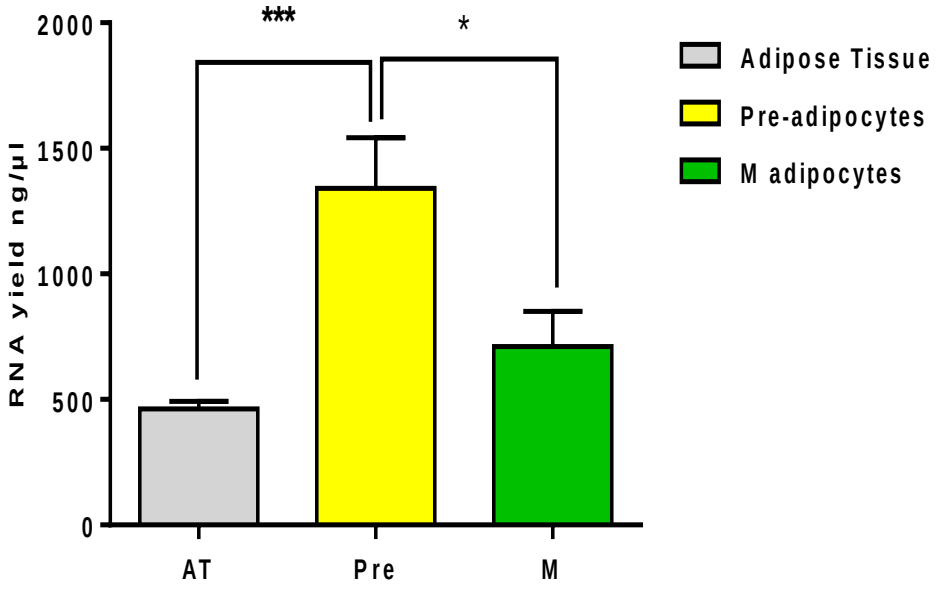


Figure 3.26 Differences in the concentration of extracted RNA from different sources. Determine the difference in the concentration of extracted RNA from adipose tissue (**AT**) (n=8), human pre-adipocytes (**Pre**) (n=5) and *in vitro* differentiated mature adipocytes (**M**) (n=5). Data presented as mean ± SEM. *** P<0.001, * P <0.05.

With regards to the purity, **fig 4.27** shows there is no significant difference in the purity of RNA extracted between different sources P >0.05.

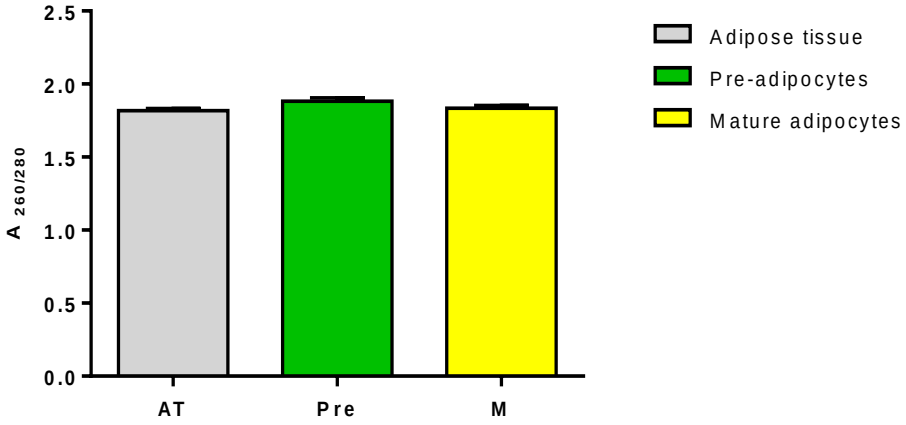


Figure 3.27 purity of RNA isolated from cell culture. There is no significant difference in the purity of RNA among cell cultures. AT - adipose tissue (n=8), pre - pre-adipocytes (n=5), M - mature adipocytes (n=5).

3.4.1.5 Discussion

Isolation of adipocytes from adipose tissue gives an opportunity to study the signalling pathways involved in the biology and in the pathophysiology of adipose tissue. Although there are a number of cell lines, such as 3T3-L1 cells, used for this purpose - these cells are aneuploid and derived from embryo fibroblasts instead of pre-adipocytes (Green and Kehinde 1975). Primary culture from adipose tissue can be isolated and *in vitro* differentiated to be used for studying the biology of adipose tissue. However, these cells are characterised by a limited lifespan, and are not always suitable for molecular studies (Debevec, Christian et al. 2007). Therefore, one of the aims of this study was to generate a primary cell culture system suitable to study the steroidogenic pathway in adipose tissue of women with PCOS.

Initially, the 3T3-L1 mouse cell line was used to optimise the protocol of culturing and differentiating adipocytes (Nestler, Clore et al. 1989)**.** The aim from starting work on this cell line was to gain skills in cell culture; to use it as a basis for validation of the protocol; and then to transfer to human primary cultures. The 3T3-L1 cell line was cultured and maintained successfully.

The protocol of differentiation was adapted from that of (Chen, Riley et al. 1996) using hormonal cocktail: insulin, dexamethasone and IBMX. The combination of dexamethasone and IBMX acts as an induction agent to stimulate differentiation. In some cases, the differentiation starts simultaneously and this suggests it may be related to the existence of factor/factors in the media, which may act as triggers for differentiation. Differentiation was achieved, with the formation of mature adipocytes and observation of enlarged lipid droplet formation. After 6 weeks of differentiation, de-differentiation happened, followed by cell death. This might be related to the release of TNFα, which is involved in cell regulation and apoptosis (Smas and Sul 1995).

Adipose tissue of women with PCOS is associated with changes in the morphology and function of adipocytes (Ntambi and Young-Cheul 2000). Accumulation of abdominal/visceral fat results in enlarged adipocytes and decreases in serum adiponectin (Mannerås-Holm, Leonhardt et al. 2010);

Hiragun et al., 1985). Although published data completing differentiation of adipocytes among normal and PCOS adipocytes are limited, some research has examined this area. Danforth et al. (2000) suggested that in obese patients, the inability of pre-adipocytes to differentiate into adipocytes might be due to excess energy intake and this may relate to metabolic dysfunction (Blouin, Veilleux et al. 2009); Danforth et al., 2000). The adipose tissue expandability hypothesis and lipodystrophy were discussed in detail in the introduction chapter.

This proposal has been supported by Yang and Aiping's (2008) observation on adipocytes in obese and non-obese subjects. He found that the expression of genes associated with differentiation, such as aP2 and adiponectin decreased (Yang and aiping 2008). Furthermore, the exposure of adipocyte to androgen interferes with differentiation of the cells. It was detected that a reduction in differentiation and lipid accumulation in normal adipocytes was associated with exposure to DHT - found to be high in PCOS women (Rebuffe-Scrive et al., 1987; (Chazenbalk, Singh et al. 2013).

Our result supports this proposal, where we have shown that pre-adipocytes (immature cells) after isolation from fresh adipose tissue have the ability to maintain their phenotype even after prolonged culturing, and retain the ability to differentiate into adipocytes. However, the rate of differentiation of adipocytes of PCOS is significantly lower than non-PCOS. This can be interpreted by suggesting that as the adipocyte environment is a complex environment consisting of several factors, most likely androgen may interfere with the adipogenesis. Indeed, several studies have determined that SAT has a higher rate of androgen production (Belanger, Hould et al. 2006). However, comparisons of the level of androgen secretion between non-PCOS and PCOS adipocytes was not performed. Research on the abnormalities in adipose tissue in PCOS is still a new area of investigation.

It is suggested that the rate of androgen synthesis from precursors and androgen inactivation in adipose tissue samples taken from SAT is higher than that taken from omental adipose tissue (Belanger et al., 2006). However, the difference in the level of androgen production between PCOS and non-PCOS has yet to be determined.

Although the Oil Red O stain was used to assess differentiation, and this is used as a marker of differentiation of adipocytes, the suitable method for this experiment is to use an alternative method to assess the maturation with other biochemical examinations measuring the level of glycerol 3-phosphate dehydrogenase (GPDH) level (Sweeney et al., 2002).

The protocol for culturing primary mature adipocytes was optimised. However, the risk of cell lysis and detachment is quite common with adipocytes, due to a lack of access to the available nutrient within the media as a result of the filling of adipocytes with the lipid. This also makes the cells easy to rupture during manipulation or washing (Barr et al., 1997). This problem was resolved by decreasing the washing step, and by avoiding use of a vacuum during media change, instead pipettes are used to take just half of the media (Invitrogen. com).

Adipocytes and their precursors exhibit individual properties based on the body region from which the cells develop. This was confirmed, in lipodystrophy, where fat shows dystrophic reactions only in a specific body part. Also, in the flap transferred process, when a flap of adipose tissue is transferred from one region to another, it will maintain the properties of the donor site (Hemmrich, Denecke et al. 2010). For clinical examination, this is of great importance, as there is evidence that regional fat distribution has a role in multiple and related diseases, such as diabetes and hypertension (Bouchard, Bray et al. 1990). Isolation of adipocytes from SAT gives an opportunity to study the signalling pathways involved in the biology and pathophysiology of adipose tissue in women with PCOS.

To understand the pathological mechanism behind the development of hyperandrogenism and insulin resistance in PCOS, and the role of adipose tissue in this disorder, gene expression profile analyses are required to reveal those genes responsible for this complex disorder. Gene analysis would require a reasonable amount of RNA isolated from adipocytes with good quality to conduct q real time PCR technique. Therefore, we need a simple, reliable and effective protocol.

Although there are several protocols used to isolate RNA from adipocytes, these result in RNA that is partially degraded and/or of poor yield or

the small RNAs are lost in the process. Due to high content of lipid in adipocytes, obtaining high quality RNA is a significant challenge.

Therefore, misleading results could be gained by using unsuitable RNA. This is a significant problem, especially when working with a limited amount of tissue (i.e. human tissue) (Janke, Engeli et al. 2001). Several technical problems yield unacceptable results; as such RNA isolation from adipocytes needs to be optimised to ensure high amounts of RNA and guaranteed RNA integrity; therefore, additional steps and modifications are necessary (Bouchard, Bray et al. 1990, Janke, Engeli et al. 2001).

Our findings demonstrate a significantly higher RNA yield with good homogenisation of the cells by: using the recommended quantity of Trizol reagent; incubation of the lysate at 37°C; repeatedly passing the lysate through pipettes; and centrifugation to remove the lipid layer - providing isolated RNA of good quality and integrity. Optimised procedures giving acceptable results will facilitate genetic studies on adipose tissue applying the qRT-PCR technique.

Even though there is difficulty maintaining the *in vivo* function of primary mature adipocytes in culture, it is possible with optimisation to produce a culture system to mimic and represent the *in vivo* function of these cells (Prins, Niesler et al. 1997).

In summary, we have generated an *in vitro* model of adipocytes of adipose tissue of women, with and without PCOS, after overcoming several limitations. Its performance in applying to PCR analysis has also been validated. Therefore, this cell system has the ability to facilitate studies of adipose tissue and enable investigation of the molecular biology of important potential factors involved in adipocyte function in non-PCOS subjects, and dysfunction in PCOS.

3.4.2 Impact of hormones (insulin ± LH) on the mRNA expression of steroidogenic enzymes (CYP17A1, AKR1C3) in adipocytes

3.4.2.1 Introduction

It is known that an increase in the androgen levels in peripheral blood of PCOS, are thought to come from high androgen production in the ovaries and adrenal glands (Franks 1995). However, there is evidence confirming the significant contribution of peripheral steroidogenesis to androgen synthesis; with the mechanism involving up-regulation of steroidogenesis in peripheral tissues (Stewart, Shackleton et al. 1990, Fassnacht, Schlenz et al. 2003). Taponen and his colleagues found that not only in women with PCOS, but also in women with simple obesity, there is a positive correlation between serum androgen levels and BMI (Taponen, Martikainen et al. 2003).

It is clear that there is a strong relationship between hyperinsulinaemia and hyperandrogenaemia in PCOS. However, there is debate about the nature of this association, and to which extent high insulin levels can produce excess androgen and how excessive androgen levels themselves stimulate insulin resistance. The question regarding the stimulation of androgen production by insulin is still not answered. For example, in women with PCOS, why does hyperinsulineamia result in high androgen, but not in patients with type 1 DM taking exogenous insulin (Zacur 2001)?

In addition, in PCOS, androgen excess facilitates insulin resistance by enhancing deposition of abdominal fat (Cascella, Palomba et al. 2008). The relationship between these two main characteristics of PCOS (hyperinsulinemia, hyperandrogenemia), especially regulation of androgen secretions in adipose tissue, is still not extensively studied (Corbould, Bawden et al. 2002, Quinkler, Sinha et al. 2004) (Killinger, Perel et al. 1990). The current understanding in the mechanism of insulin-induced hyperandrogenemia in PCOS is limited, and needs further work.

The aim of the work described in this chapter was to investigate the action of insulin ± LH on mRNA expression of the androgen metabolism

enzymes, (CYP17A1, and AKR1C3) from women without PCOS and women with PCOS.

3.4.2.2 Objectives

The objectives of this section of the research were:

- To investigate the impact of Insulin on the expression of steroidogenic enzymes, CYP17A1 and AKR1C3 in *in vitro* differentiated monolayer cultures of adipocytes from women with and without PCOS, using RT-PCR technique.

- To study the impact of LH alone and with insulin on the expression of CYP17A1 and AKR1C3 in adipocytes of both groups (non-PCOS, and PCOS)

- To examine the role of the PI3-K signalling pathway in insulin regulation of testosterone synthesis in peripheral adipose tissue of women with and without PCOS.

3.4.2.3 Methods

Methods were described in detail in **Chapter 2.**

3.4.2.4 Results

3.4.2.4.1 Effect of increasing concentrations of insulin on CYP17A1 mRNA expression in adipocytes

The experiments demonstrated that there are highly significant (P=0.0001) differences in the basal level of the expression of CYP17A1 between adipocytes isolated from adipose tissues of PCOS compared with non-PCOS (**fig 3.28**). The basal normalized level of CYP17A1 mRNA (ΔCt) in non-PCOS (n=5) was 1.78± 0.68, whereas in PCOS women (n=5) was 17.78± 1.30. Of the reference genes tested, GAPDH, ACTB, and LPR10 were shown to be consistently expressed across PCOS and control groups (P>0.05). The expression of the gene in PCOS women was raised approximately ten-fold above the non PCOS samples.

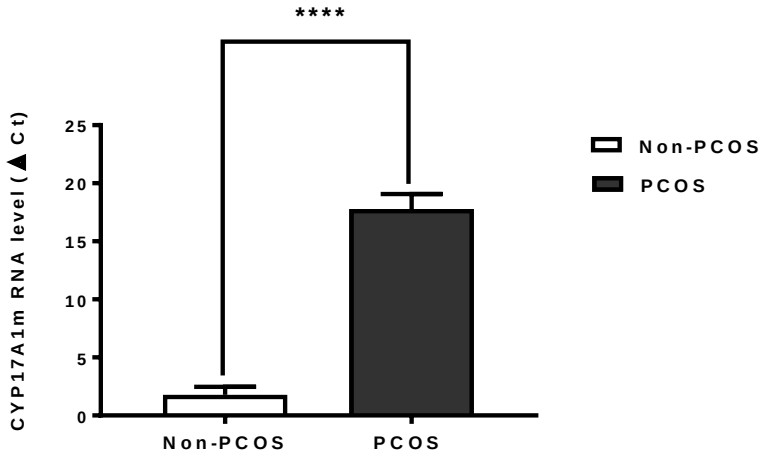


Figure 3.5 Difference in the basal level of CYP17A1 mRNA in *in vitro* differentiated adipocytes of non-PCOS and PCOS women. *In vitro* differentiated monolayer adipocyte cultures of non-PCOS (n=5) and PCOS (n=5) were used - after an overnight serum-free period - to measure the mRNA levels of CYP17A1 in adipocytes by qRT-PCR technique. 0 means no treatment. Total RNA was extracted from cultured cells by the Trizol reagent method and after assessment and c DNA synthesis, qRT-PCR was carried out; expression of the target gene was normalised to validate consistently expressed reference genes (GABDH, ACTB, and LRP10). Data presented as (mean± SEM). ****P <0.001.

With regard to the impact of increasing concentrations of insulin on the expression of CYP17A1 of non-PCOS women compared with PCOS adipocytes, there was no significant (P>0.05) change in the CYP17A1 mRNA normalized level in adipocytes of both groups to any of the three concentrations of insulin (0, 1, 10, and 100nM) (**fig 4.26**). Data were analysed by using multiple comparison one-way ANOVA test (**see section 2.14**)

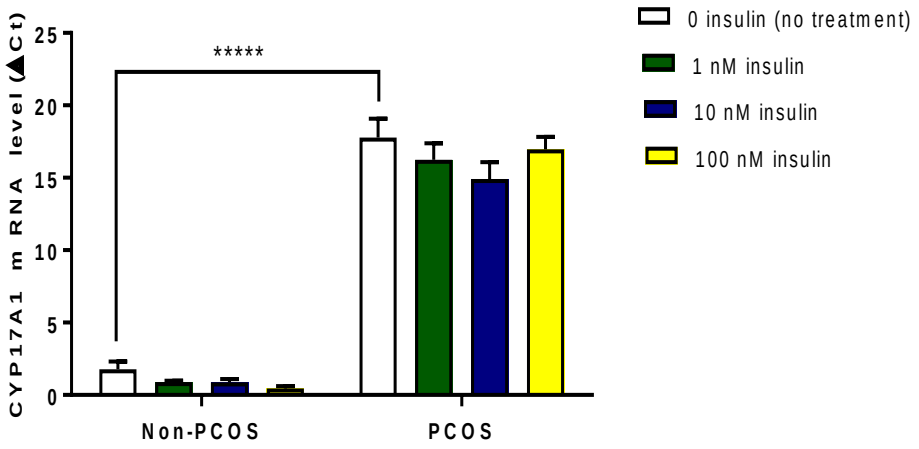


Figure 3.6 Difference in the expression of CYP17A1 mRNA between non-PCOS and PCOS under the effect of different concentrations of insulin. An *in vitro* differentiated monolayer of mature adipocytes from normal women (n=5) and women with PCOS (n=5) were cultured - after overnight serum free period - for 72hrs under the treatment of different concentrations of insulin (0, 1, 10, and 100 nM). 0 is no treatment; serum free medium only. Total RNA was extracted from the monolayer cell culture using Trizol reagent method and the expression level was measured using qRT-PCR and normalised to the three reference genes (GAPDH, ACBT, and LRP10). Data was presented as mean ±SEM. (ΔCt) **** P <0.0001.

3.4.2.4.2 Impact of Insulin +LH on CYP17A1 mRNA expression in adipocytes

The results of the experiments showed that in non-PCOS women, LH only has a significant effect at one concentration (10nm/l), (P=0.0003) on mRNA expression of CYP17A1. The basal level of CYP17A1 is (1.12±1.2), with LH treatment increasing it to (14.05±1.1) (**fig 4.27)**. However, in PCOS, LH had no significant effect on the expression of this enzyme (P= 0.99) - the basal level of CYP17A1 was 17.78±1.3, with LH 18.68±1.1 (**fig 4.27**).

Regarding the impact of increasing concentrations of insulin with LH, the results show that in both groups insulin had no additional significant effect (P>0, 05) on the expression of CYP17A1 **fig 3**. The mean± SEM of normalised CYP17A1 mRNA in non-PCOS is (LH+1nm) (LH+10nm) (LH+100nm) (13.63±1.1) (12.92±0.6) (6.58±0.7) respectively; in PCOS was (17.08±0.8) (17.09±0.79) (13.93±1.2) **table 3.6.**

In this experiment only one concentration of LH, 10nm/L, was used. Based on published data, physiological levels of LH on regulation of steroidogenesis in adipocytes. Little is known concerning the role of LH on adipose tissue steroidogenesis metabolism - especially in women with PCOS.

	Non-PCOS(n=5) mean± SEM	PCOS(n=5) mean± SEM
No-treatment (no insulin or LH	1.12±1.2	17.78±1.3
LH (10nm/l)	14.05±1.1	18.68±1.1
LH+1 nm/l	13.63±1.1	17.08±0.8
LH+10 nm/l	12.92±0.6	17.09±0.79
LH+100 nm/l	6.58±0.7	13.93±1.2

Table 3.6 shows the normalized level of CYP17A1 mRNA of *in vitro* differentiated adipocytes of non-PCOS (n=5) and PCOS (n=5) under the effect of increasing concentrations of insulin and only one concentration of LH.

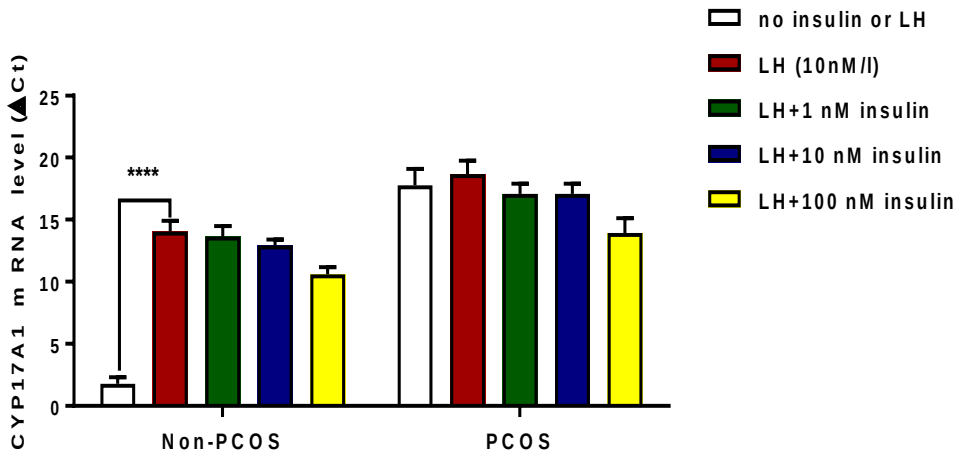


Figure 3.31 CYP17A1 mRNA expression after treatment with insulin and LH in adipocytes of non-PCOS and PCOS women. *In vitro* differentiated adipocyte cells were cultured for 72 hr with increasing concentrations of insulin (0, 1, 10, 100nM) ± LH 10nM after 24 hrs of serum free medium. Total RNA was isolated from the cell culture monolayer. After RNA assessment and c DNA synthesis, CYP17A1 mRNA was measured by qRT-PCR, the reference genes GAPDH, ACTB, and LPR10 were used for normalisation. Data presented as the mean± SEM; a significant effect was seen from 10 nM LH (**** P<0.0001), and from LH with all different concentrations of insulin (1, 10, 100n M) (****P<0.0001) in non-PCOS adipocytes.

3.4.2.4.3 Impact of increasing concentrations of insulin on AKR1C3 mRNA expression in adipocytes

Studying the effect of increasing concentrations of insulin (0, 1, 10, and 100 nM) on the expression level of AKR1C3 mRNA in adipocytes of non-PCOS women (n=5) showed that the expression level of AKR1C3 significantly increased with increasing concentrations of insulin **tab 3. 7** (**fig 3.32**).

However, in women with PCOS, the study showed that there is no significant change in the basal level of AKR1C3 mRNA levels with different concentrations of insulin. P >0.05 (**fig 3.32**).

In order to reveal the significant and a big difference in the expression level of AKR1C3 mRNA under the action of different concentrations of insulin between adipocytes of normal women and women with PCOS, we made a comparison between it. The data shows that the basal level of AKR1C3 mRNA in adipocytes of PCOS is significantly abundant than that of non-PCOS women, P<0.05(P =0.01), **fig 3.33.**

Insulin concentrations(nm/l)	AKR1C3 mRNA level mean± SEM (ΔCt)
(0) No treatment	0.967±0.21
1nm	1.593±0.84
10nm	2.399±1.23
100nm	7.421±0.85

Table 3.7 shows ALKR1C3 mRNA level of non-PCOS (n=5) under the action of increasing concentrations of insulin. Insulin upregulates AKR1C3 mRNA expression in adipocytes in non-PCOS (n=5).

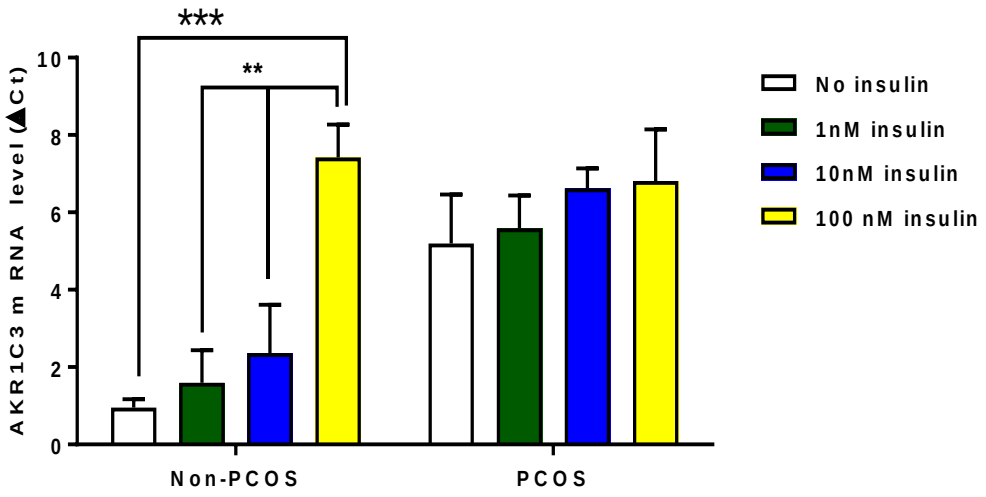


Figure 3.32 shows the difference and alteration in the basal level of AKR1C3 mRNA expression under the impact of increasing concentrations of insulin between adipocytes of non-PCOS (n=5) and PCOS (n=5). Illustrates the highly significant effect of high concentration of insulin (100nm) on AKR1C3 expression in adipocytes of normal women, P=0.0003. Data presented as mean± SEM.

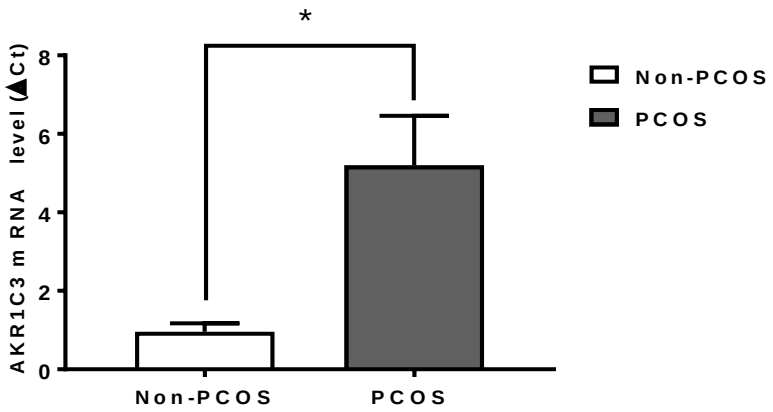


Figure 3.33 Comparison of basal levels of AKR1C3 mRNA between adipocytes of women with and without PCOS. It demonstrates the significant difference in the basal level of AKR1C3 mRNA level between two groups, ** p =0.01, data presented as mean± SEM.

Using in vitro differentiated adipocytes (a study design that enabled us to study possible effects of insulin on AKR1C3, we demonstrated that changes in insulin levels that span the physiological range (pre-or postprandially) 100-2000 pm/l (Basu 2011) had significant effects on non-PCOS.

3.4.2.4.4 Role of PI3-Kinase inhibitor in insulin action on AKR1C3 expression

Based on the previous results of this study - which explored the significant effect of insulin in high concentration on the expression of AKR1C3 in adipocytes of non-PCOS women (**section 3.3.2.4.3, Fig 3.32**) - we investigated the impact of application of a selective insulin pathway inhibitor (LY292004) which blocks the PI3Kase pathway on AKR1C3 expression in the presence of insulin; in order to understand the molecular mechanism of insulin on the steroidogenic pathway, particularly on AKR1C3 expression in adipocytes of non-PCOS women.

The experiment showed that with PI3Kase inhibitor (LY294002), there is no significant effect of insulin on the expression of AKR1C3 in adipocytes of non-PCOS, $P > 0.05$ (**fig 3.34**).

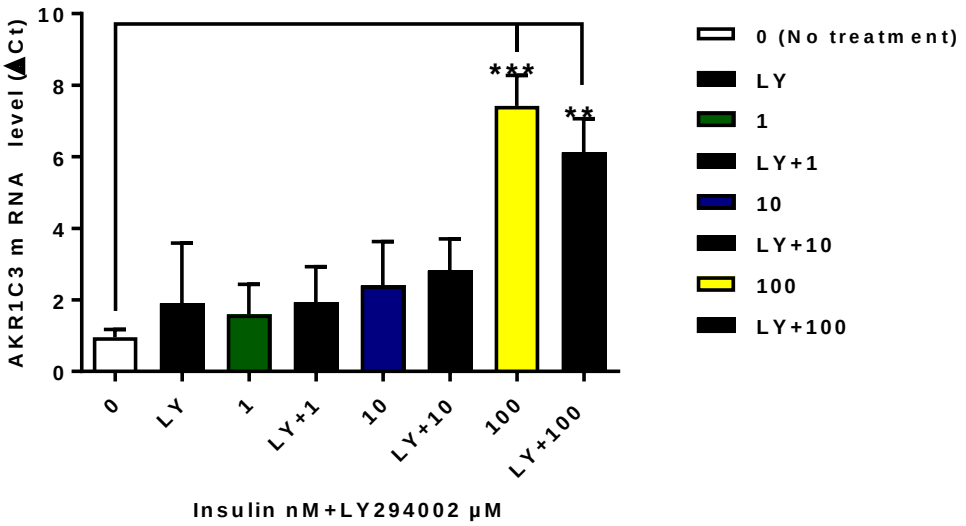


Figure 3.34 demonstrates the impact of a selective insulin pathway inhibitor (LY294002) on AKR1C3 mRNA expression in adipocytes of non-PCOS women. *In vitro* differentiated adipocytes of non-PCOS (n=5) treated with insulin ±PI3-K inhibitor (LY294002) at 10µm/dl. Results are mean ± SEM. 0 concentration means no treatment - which is a media free from any treatment and used in the experiment as a control. Significance was seen at 0 nM/100 nM, *** P =0.0003 and with blocking, the effect of insulin remains significant, 0 nM/LY+100n M** P =0.004.

3.4.2.4.5 Impact of insulin +LH treatments on the expression of AKR1C3 in adipocytes

The data of our study show that LH without insulin significantly upregulates (P = 0.005) the expression of AKR1C3 in non-PCOS adipocytes. However, in the presence of insulin (insulin+ LH), the mRNA level of AKR1C3 in adipocytes of non-PCOS did not alter (**fig 3.35**). LH enhances AKR1C3 expression in non-PCOS adipocytes independent of insulin.

In PCOS, there is no significant impact of LH, or insulin with LH - on the AKR1C3 expression, P value>0.05 (**fig 3.53**).

LH stimulates AKR1C3 in non-PCOS adipocytes independent of insulin. However, in PCOS adipocytes, LH has no impact on AKR1C3 mRNA expression. This suggests that a high dose of LH is needed to stimulate its receptor in PCOS adipocytes, or maybe there are molecular modulators in the adipocyte's environment that downregulates LH receptors or resists the action of LH and insulin, or may just that expression is already maximal.

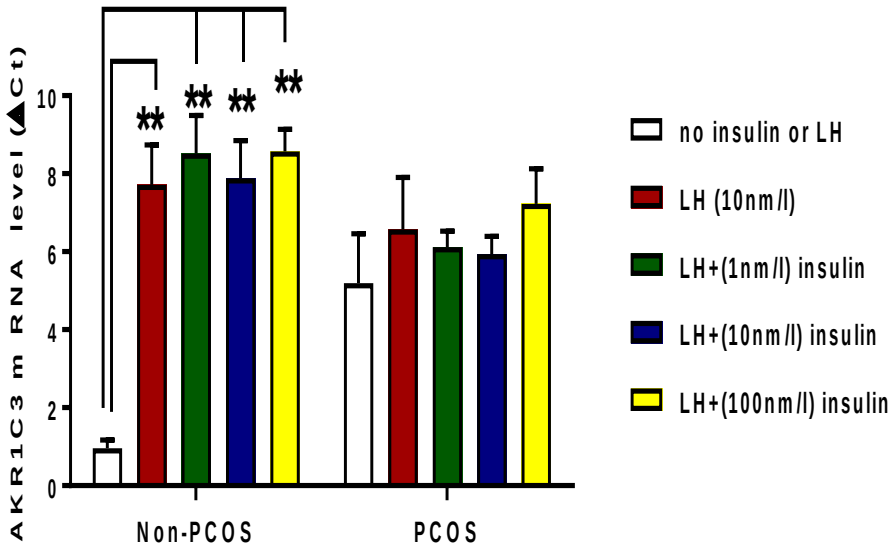


Figure 3.35 demonstrates the impact of insulin (nM) ±LH (10 nM) on the expression of *in vitro* differentiated adipocytes of women without (n=5) and with PCOS (n=5). Adipocytes were treated with increasing amounts of insulin (1, 10, and 100), and 10 nM of LH, to investigate the expression level of mRNA of AKR1C3. 0 means no treatment at all (no insulin or LH, and was used as a control in this experiment). RNA was extracted from cell culture monolayer cells by Trizol reagent method; after assessment the expression level of target enzymes was quantified using qRT-PCR technique normalised with consistently expressed reference genes (GADH, ACTB, and LPR10). Data expressed as means± SEM. Significance was observed at 0, no any treatment, 10 nM of LH **P =0.005, and at 0 nM/ 10 nM of LH+1, 10, and 100 nM of insulin ** P <0.01.

3.4.3 Action of insulin± LH on testosterone production from adipocytes

3.4.3.1 Introduction

The observations and results of the previous part of the study suggest differences in the basal level of CYP17A1 and AKR1C3 mRNA expression between non-PCOS and PCOS adipocytes, and in non-PCOS AKR1C3 expression showing increased sensitivity to insulin treatment. These also showed CYP17A1 and AKR1C3 expression in response to LH treatment in non-PCOS adipocytes. We decided to investigate whether the levels of treatment used in our previous experiments would affect our detection of testosterone secretion.

3.4.3.2 Objectives

- To investigate the impact of increasing concentrations of insulin on testosterone production from adipocytes of women, with and without PCOS.

- To study the impact of LH at only one concentration (10nm/l) alone, and with insulin - on testosterone production from adipocytes of non-PCOS and PCOS women.

- To investigate the molecular mechanism of insulin by studying the role of the PI3-K signaling pathway in insulin regulation of testosterone synthesis in adipocytes of women with and without PCOS - by application of PI3-K ase inhibitor, LY294002 at a concentration of 10µm/dl.

3.4.3.3 Methods

Culture media of *in vitro* differentiated adipocytes was used to measure the secretion of testosterone using ELISA after treating the culture with a range of treatments (recombinant insulin +/-LH+, 10nm/l/-PI3-Kase inhibitor (LY294002, 10µm/dl). Non-PCOS mature adipocytes were cultured from SAT biopsy samples (n=5), and matched with PCOS samples (n=5) for age and BMI, **see tab2.1**. After maturation and various treatments, supernatant was collected for testosterone measurement before and after treatment. Enzyme-linked immunosorbent assay (ELISA) technique was used to satisfy the aims of this part of the study. It was described in detail in **chapter 2, section 2.13**.

3.4.3.4 Results

3.4.3.4.1 Impact of increasing insulin concentrations on non-PCOS and PCOS adipocyte testosterone secretion

After creation of the standard curve, **see section 2.13.2, fig 2.13**, we measured testosterone secretion from our *in vitro* differentiated adipocytes of two groups, non-PCOS (n=5) and PCOS (n=5). **Fig 3.36** shows the secretion of testosterone from non-PCOS adipocytes significantly increased under various concentrations of insulin (0, 1, 10, 100 nM) (0 means no insulin to be used as a control), ; the effect seemed to be dose-dependent - with increasing testosterone secretion at 1nM (P=0.003), 10nM (P=0.0001) and at 100 nM (P<0.0001) when compared to no treatment.

However, for PCOS adipocytes, **fig 3.37** shows that there is no significant effect of insulin at different concentrations on testosterone secretion from adipocytes of PCOS, P >0.05.

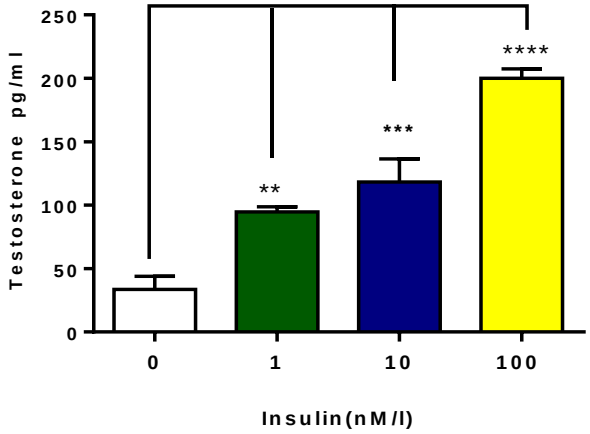


Figure 3.36 testosterone secretion (pg/ml) by non-PCOS adipocytes after insulin treatment at different concentrations. After a 24hr free serum period, Non-PCOS mature adipocytes were treated with different concentrations of insulin (0, 1, 10, 100 nM) for 3 days; and the media was collected and examined for testosterone levels by ELISA technique. All testosterone concentration measurements were compared to control (0, no treatment). Testosterone significantly increased with increasing doses of insulin (1nM, **P=0.003) (10nM, ***P=0.0001) and (100 nM, **** P<0.0001), examined using one-way ANOVA test.

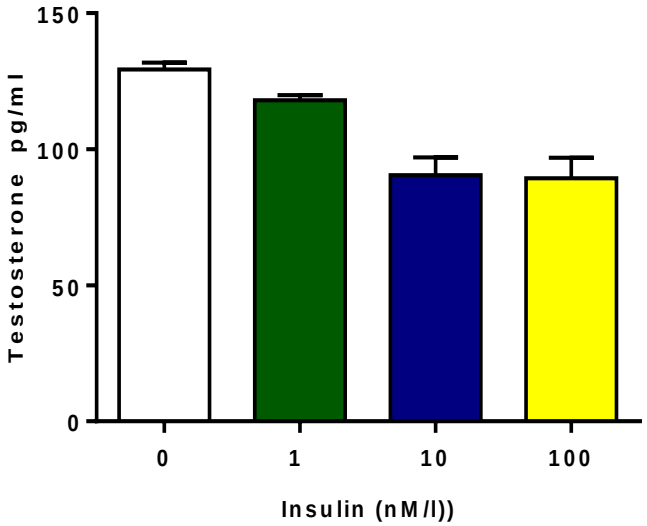


Figure 3.37 the impact of various insulin treatments on testosterone secretion from PCOS adipocytes (n=5). *In vitro* differentiated adipocytes of PCOS (n=5) were treated with various concentrations of insulin (0, 1, 10, and 100nM). Supernatant was collected and used to measure testosterone secretion by using ELISA. Data was analysed by using a one-way ANOVA test to compare testosterone levels. There was no significant effect of insulin (P>0.05). Data presented as mean± SEM.

We then compared with the basal level of testosterone secretion from our primary culture of non-PCOS and PCOS adipocytes, **fig 3.38** shows significant variation, P <0.0001 in the basal level (no treatment) of secreting testosterone between two groups.

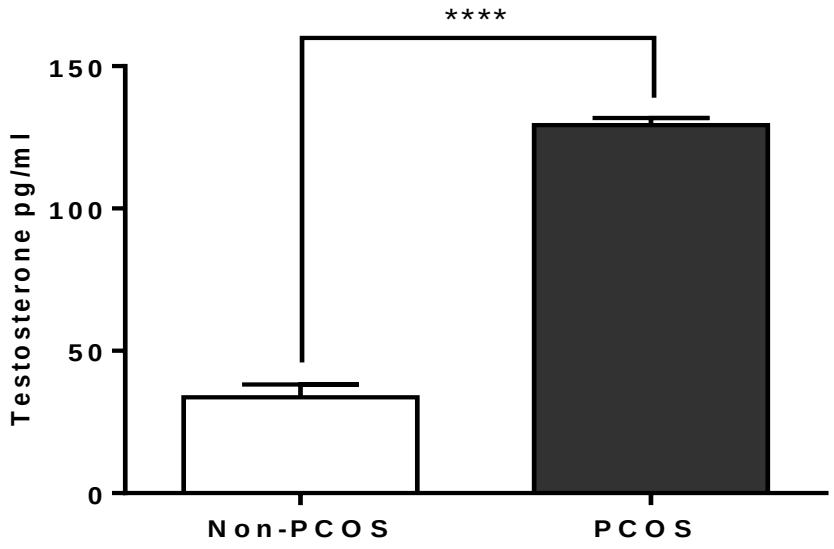


Figure 3.38 comparison of basal levels of testosterone secretion of non-PCOS and PCOS adipocytes. Comparison of basal levels of testosterone secretion of non-PCOS adipocytes (n=5) and PCOS (n=5) after collection and measurement from non-treated primary culture (no insulin). There is highly significant difference in the androgen level ****P <0.0001.

The possible effect of insulin on testosterone secretion in non-PCOS adipocytes may therefore indicate it has a role in androgen synthesis. We investigated a possible signalling pathway by blocking PI3-K, which was observed to be involved in ovarian steroidogenesis (Munir et al. 2004). **Fig 3.39** shows that no significant variation in non-PCOS adipocytes was observed whether through insulin stimulation of androgen secretion or by inhibition of PI3-K pathway. Adding PI3-K inhibitor (LY294002, 10ng/ml) to insulin did not change the magnitude of insulin effects on testosterone concentrations in non-PCOS adipocyte culture supernatant

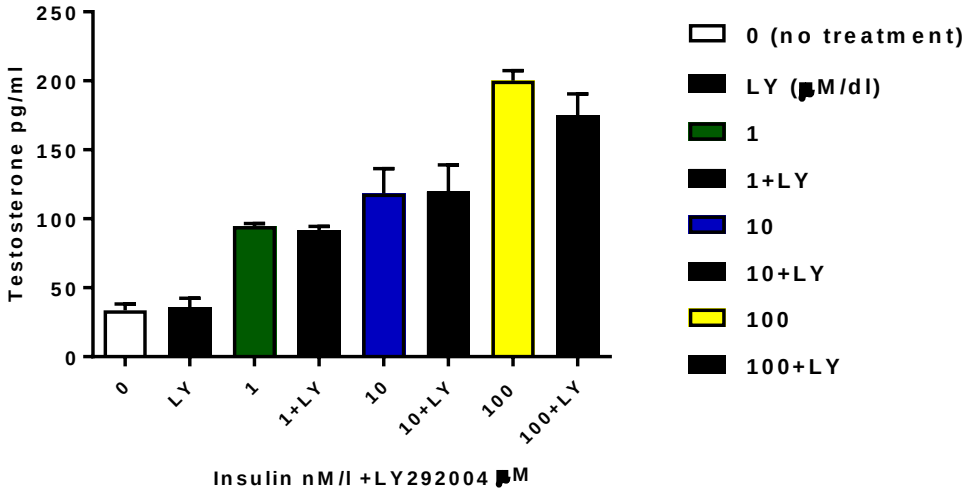


Figure 3.39 shows non-PCOS testosterone secretion with and without inhibition of the insulin signalling pathway. Non-PCOS adipocytes (n=5) were treated with insulin, with and without the PI3-K inhibitor (LY292004 (10µm/dl)) - and testosterone secretion measured. No significant variation was seen between treated and untreated culture with a signalling pathway inhibitor. Results are mean± SEM.

3.4.3.4.2 Effects of LH on non-PCOS and PCOS adipocyte testosterone secretion

In ovarian theca cells the secretion of androgen increases with augmentation of LH and insulin (Gilling-Smith, Willis et al. 1994); (Munir, Yen et al. 2004). In this study, we treated the non-PCOS and PCOS cultures with a combination of insulin and LH, and its effect on the level of testosterone secretion was measured.

The increase in testosterone levels with increasing insulin dose in the non-PCOS adipocyte culture supernatant followed a dose dependent fashion. The increase in testosterone concentrations in non-PCOS adipocyte culture supernatant was marked when LH was added alone or with insulin. We found a significant increase in testosterone secretion among treatments with LH (**** $P < 0.0001$), **Fig 3.40**.

However, in the PCOS group, the experiments showed that there was no significant change ($P > 0.05$) in testosterone secretion throughout treatment with LH± insulin **fig 3.41.**

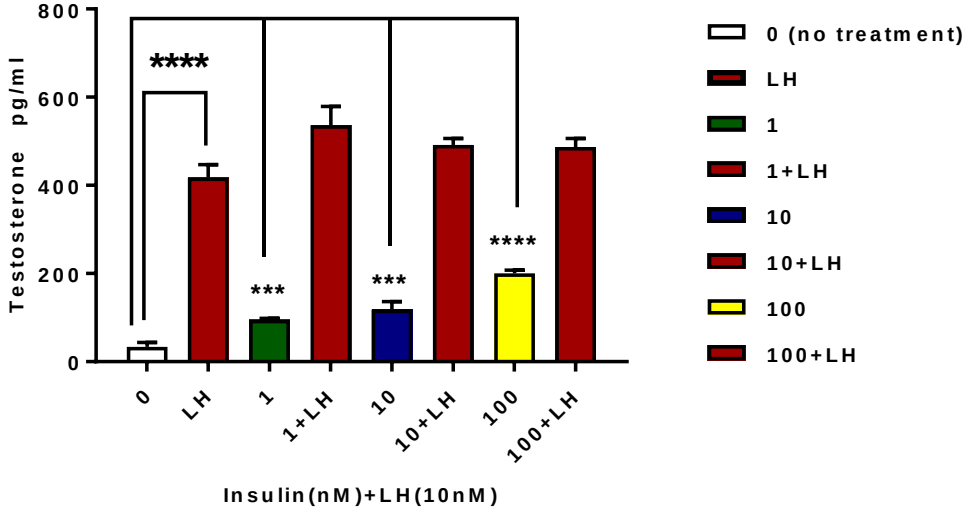


Figure 3.40 Non-PCOS mature adipocyte testosterone secretion with LH and insulin treatments. LH± insulin treated non-PCOS (n=5) media was examined for testosterone using ELISA technique. Results are mean± SEM; all levels were highly significant against untreated (0 non-treatment) and LH treated culture. **** P <0.0001.

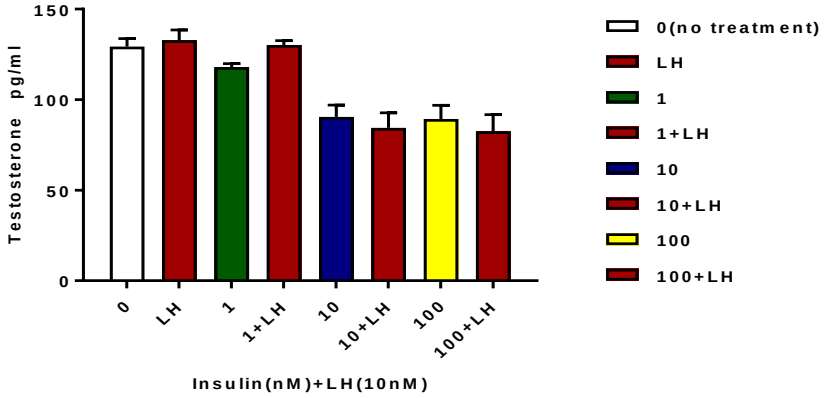


Figure 3.41 Shows the impact of LH± insulin on testosterone secretion from PCOS (n=5) adipocytes. Supernatant of adipocytes treated with insulin ± LH was collected and investigated for testosterone using ELISA technique. There was no significant change in testosterone secretion. Results presented as mean± SEM. P >0.05.

3.4.4 Discussion

In order to study the relationship between insulin and androgens; the role of adipocytes in the pathogenesis of PCOS; and to understand the causal effect of insulin in the pathophysiology of PCOS, we investigated the impact of insulin on the expression of the two main enzymes in steroidogenesis at the cellular level - using an *in vitro* differentiated adipocyte culture not previously used for PCOS women.

Our data indicate that adipocytes are likely to perform androgen biosynthesis from a main precursor - cholesterol, because the mRNAs of the key steroidogenic enzyme CYP17A1 have been demonstrated in human primary adipocytes. This suggestion is consistent with the report by Puch and co-workers (2002), but the difference between our study and that report is that this study performed the investigation at the cellular level using primary cell culture; while they investigated at a tissue level. In addition, recently in 2014, Cadagan et al. found that pre and mature adipocytes express CYP17A1 as detected by WB, and they located this enzyme within adipocytes (Cadagan, Khan et al. 2014). These data support the findings of this study regarding CYP17A1. Currently, adipocytes are known to be involved in steroidogenesis (Simpson 1999); (Wildes, Marcus et al. 2010); (Harlass, Plymate et al. 1984). However, until now, this was suggested to utilise circulatory precursors. In fact, our investigation in this field will therefore help the current information on adipocytes metabolism.

In addition, data from the study supports an intracrine role of adipocytes as the terminal activator of inactive androgen precursors (androstenedione) into active sex steroids (testosterone). The detection of AKR1C3 mRNA confirms previous findings on the occurrence of testosterone synthesis in adipose tissue, as reviewed by Belanger and his colleagues (Belanger, Luu-The et al. 2002). In 2009, Blouin compared human omental and SC samples for the activity of steroidogenesis in premature (pre-adipocytes) and mature adipocytes. They observed that the activity and expression of the steroidogenic enzymes, AKR1C3, P450-aromatase, steroid sulphatase, ERα and 17β-HSD-3 are

elevated after differentiation or formation of mature adipocytes of subcutaneous adipose tissue (Blouin, Nadeau et al. 2009).

Currently, adipocytes are recognised to be involved in the steroidogenic pathway through androgen and oestrogen modification (Blouin, Veilleux et al. 2009); (Simpson, Rubin et al. 1999, Simpson 1999). Pedersen et al in 1996 detected the existence of androgen receptors in both pre-adipocytes and mature adipocytes; and in common with our finding - may also support the presence of an androgen pathway in adipocytes (Pedersen, Fuglsig et al. 1996); (Anderson, Lavoie et al. 2001); (Chazenbalk, Singh et al. 2013). By detecting mRNA of CYP17A1 in adipocytes, we support the significant regulatory role androgen may have in localised production and synthesis of androgen.

Further to the detection of CYP17A1 expression in adipose tissue of women with and without PCOS - and the determination of significant expression of AKR1C3 in PCOS adipose tissue; this study demonstrates that the basal level of two enzymes, CYP17A1 and AKR1C3, are greater in *in vitro* differentiated adipocytes of women with PCOS in comparison with non-PCOS women; and that the expression of CYP17A1 mRNA and AKR1C3 mRNA are higher in adipocytes of women with PCOS. This may suggest local high androgen production and may help to explain the relationship between adipose tissue, obesity and features of high androgen. The evidence supports the significant contribution of peripheral steroidogenesis to androgen synthesis and excess, via mechanisms that involve up-regulation of steroidogenesis in peripheral tissues, such as adipose tissue (Fassnacht, Schlenz et al. 2003); (Stewart, Shackleton et al. 1990). (Taponen, Martikainen et al. 2003) found not only in women with PCOS but also in women with simple obesity, that there is a positive correlation between serum androgen and BMI.

Based on the previous findings of this study, and the observation of Puch and colleagues, we investigated the expression level of CYP17A1 and AKR1C3 under the impact of the most common steroidogenesis regulators, insulin and LH. The initial experiments studying CYP17A1 expression across non-PCOS and PCOS adipocytes showed that insulin has no impact on the expression of

CYP17A1 in both groups studied - as there was no change in the basal level of CYP17A1 after treatment with insulin.

Regarding AKR1C3 expression under the effect of insulin, it was observed that in PCOS adipocytes the mRNA of AKR1C3 was unchanged after insulin treatment. However, in adipocytes of non-PCOS women, we observed that insulin steadily upregulates the expression of AKR1C3 (17β-HSD); and at high concentrations it enhances the expression of this enzyme.

The possible interpretations for these findings or observations are that in non-PCOS women, serum insulin concentration is not increased and is not significantly high enough to stimulate CYP17A1; and may be the same for AKR1C3. But in women with PCOS, the prevalence of insulin resistance with the development of compensatory hyperinsulinemia which may produce its impact on mRNA expression. Also, it may be explained that the expression level is already maximal in PCOS. In 2005, Munir proposed that ovarian theca cells of women with PCOS, hyperinsulinemia leads to molecular defects in CYP17A1 expression and increased androgen production (Munir, Yen et al. 2005).

However, the other more attractive and interesting explanation for our findings is that normal women have no genetic predisposition or defect to the stimulatory effect of insulin on adipocytes. It appears likely that the presence of a PCOS gene or combination of genes, with the presence of predisposing environmental factors, may modify the adipocytes of women with PCOS, more responsive to insulin stimulation of androgen production (Dunaif 1997).This proposal may give some explanation to the observations of this study.

Our observations are consistent with the findings of *in vivo* studies, which showed that acute hyperinsulinemia by insulin infusion has no effect on circulating testosterone in non-PCOS women (Nestler, Clore et al. 1987); (Smith 1987); (Dunaif and Graf 1989).

Gene mutation could be the cause of the pathogenesis of PCOS. This mutation enhances the activity of CYP17A1. This mutation could occur in the gene encoding P450 C17, or serine phosphorylation, but until now no mutation in a single such gene has been reported (Qin and Rosenfield 1998). In PCOS,

ovarian theca cells are considered the main site of androgen secretion, as the major sites where the enzymes required for androgen biosynthesis are expressed. Moreover, it appears that local tissue factors modulate the activity of CYP17A1 (Qin and Rosenfield 1998). Indeed, with phosphorylation of serine of P450c17, the activity of lyase is enhanced. Therefore, the ratio activity of 17,20 lyase to 17 hydroxylase is increased. This may be relevant to the pathogenesis of excessive androgen levels in PCOS (Auchus, Geller et al. 1998). If protein kinase, which phosphorylates serine in the insulin receptor results in insulin resistance is the same protein as that phosphorylating serine in the P450c17 causing hyperandrogenism, this might explain the relationship between insulin resistance and androgen excess in PCOS by a single gene defect (Qin and Rosenfield 1998).

Genetic causes in the pathogenesis of PCOS have been suggested in familial PCOS (Prapas, Karkanaki et al. 2009), such as dysregulation of P450 c17 which might result from genetic mutation. However, evidence to support the genetic basis of hyperactivity of this gene is limited (Qin and Rosenfield 1998).

Insulin resistance and compensatory hyperinsulinemia in PCOS is unique and not associated with common features of insulin resistance, such as hypertension (DeFronzo and Ferrannini 1991); (Zimmermann, Phillips et al. 1992), but rather is associated with ovarian steroidogenesis. Therefore, insulin resistance in PCOS may represent a distinct phenotype. Most previous studies at the cellular level have been performed on the main steroid hormone synthesis organs, namely ovaries and adrenal gland; but not on a classic insulin target tissue such as adipose tissue. Therefore, we performed this study in order to investigate at a cellular level the effect of insulin on steroidogenesis in the major insulin target tissue. This is adipose tissue, and particularly SAT depot, after confirmation by several studies that SAT, especially the lower abdomen, has a significant role in insulin resistance and disturbance in the serum lipid profile in PCOS (Wehr, Moller et al. 2009).

In 1992, Dunaif found that insulin resistance in PCOS is more profound than that which is associated with obesity (Dunaif, Segal et al. 1992). Furthermore, this was independent of changes in glucose tolerance in comparison to

NIDDM subjects (Firth, Bell et al. 1987). Insulin resistance in PCOS is intrinsic but has a different genetic basis; happens in the absence of metabolic disturbance; and is not associated with endogenous sex steroid levels (Dunaif, Segal et al. 1992)

From these observations, we postulate that insulin influences androgen metabolism in adipocytes at high concentration causing an increase in the expression of AKR1C3 mRNA in adipocytes of normal women. We took this result and examined the molecular mechanism and the possible pathway which may impact on androgen metabolism, similar to that seen in ovarian androgen formation. The protocol used was similar to that used by (Munir, Yen et al. 2004). We examined the action of insulin treatment concentrations, previously shown by application of a selective PI3Kase inhibitor (LY294002) to block the PI3Kase signal pathway, which is one of the key signal pathways of insulin action. We observed that there is no variation in the expression of AKR1C3 in the non-PCOS group, where the insulin shows its effect on the expression of the enzymes between inhibited and untreated samples. This suggests that if insulin were to be affecting androgen metabolism, it is not working through the PI3Kase pathway. Hence, many suggest other intracellular signalling pathways that may mediate insulin stimulation of adipocyte androgen biosynthesis. Another major intracellular signalling pathway which can be activated by insulin and independent of PI3-Kinase is the MAPK pathway (Withers 2000), which may be involved in adipocyte androgen biosynthesis. This pathway needs to investigate whether is involved in mediating insulin stimulation of androgens biosynthesis in adipocytes.

As mentioned above, one of the investigations of this study was examination of the molecular mechanism of insulin on the expression of AKR1C3 in normal women by application of a PI3Kase inhibitor. This showed that there was no blocking effect of insulin. There are a number of mechanisms by which insulin could affect androgen production. It is believed that in women with PCOS, insulin enhances ovarian androgen production by activating a signal transduction system, which is unique and separate from the system used to stimulate glucose transport (Nestler and Jakubowicz 1997). Two studies, one performed in human placental cytotrophoblasts (Nestler, Jakubowicz et al. 1999)

and the other in swine ovarian granulosa cells (Romero, Garmey et al. 1993), support this probability by showing that the inositol-phosphoglycan system serves as the signal transduction pathway for the impact of insulin on the steroid biosynthesis pathway in these tissues.

The significance of these findings is that the inositolphosphoglycan signal transduction system may persist intact in a condition associated with insulin resistance in terms of an impaired tyrosine kinase system and glucose transport. Therefore, using another signal transduction pathway, the effect of insulin will be maintained even in the presence of glucose intolerance. (Nestler and Jakubowicz 1997) provided evidence that insulin stimulates testosterone production by human ovarian theca cells through the insitolglycan system, which acts as a transduction system for insulin (Nestler 1997).

With regard to LH and its relationship with the effect of insulin as regulator of steroidogenesis, studies in the human theca cells - which is the main cell of steroid synthesis - have found that insulin alone has no effect on CYP17A1 expression. Indeed, it required the concomitant activation of the c AMP signalling pathway using LH analogues to enhance the expression of enzyme (Munir, Yen et al. 2004). Based on this observation and on the information from the literature, which include that women with PCOS often have abnormalities in the LH level, studies have observed that the frequency of LH pulse is increased in PCOS (Berga, Guzick et al. 1993). Other studies have established that there is no difference in the frequency of LH pulse between normal and PCOS women (Dunaif, Mandeli et al. 1988).

However, in general, the amplitude of LH pulse is increased in PCOS women in contrast to healthy women (Berga, Guzick et al. 1993). It is suggested that there is a defect in the dynamics of LH caused or enhanced by hyperinsulinemia (Nestler 1997).

These lines of evidence are supported by the findings of a study performed using metformin treatment to decrease circulating levels of insulin. This found that it was associated with a decrease in the basal level of LH or LH release (Nestler and Jakubowicz 1996). In obese women with PCOS, insulin enhances the amplitude of LH. Dos Santos in 2007 reported that human

adipocytes and their precursors express LH–receptors, which suggests that fat tissue is a target tissue for this hormone.

LH has a positive effect on the proliferation and differentiation capacities of human pre-adipocytes. It also enhances the secretion of leptin from human adipose fragments (Dos Santos, Dieudonne et al. 2007). This is consistent with the observation of Sivan et al. (1998) in fully differentiated 3T3-L1 pre-adipocytes, that LH stimulates the secretion and /or the stability of the leptin protein. This relationship could be explained by the increase in SC fat deposition in pregnancy.

Our study observed that LH has a significant effect on the steroidogenesis pathway in normal women by upregulating the two main enzymes of steroid hormone biosynthesis. However, it has no impact on the PCOS women. The interpretation for this observation is that it may suggest the presence of a defect in PCOS adipocytes. This defect is related to the sensitivity - rather than complete abolition - of the impact of LH on steroidogenesis. In future work, it may be better to investigate this again with an increased treatment range of LH to detect its actual sensitivity. (Willis, Mason et al. 1996).

In 2007, after studying SC pre-adipocytes of women with PCOS, Corbould concluded that there was no intrinsic defect of insulin signalling in adipose cell lineage, and the insulin resistance is due to the presence of factors (Corbould, Bawden et al. 2002) in the *in vivo* environment.

As the environment of adipocytes is complex due to its production, which may be an important factor in the pathogenesis of PCOS, such as adiponectin, resistin, and leptin, these were discussed previously. Puch and their colleagues observed that the activity of CYP17A1 (17α-OHase) activity was independent of the hormonal status of the women. This observation suggests differential tissue-specific regulation of the CYP17A1 gene in the ovary and adipose tissue (Puch et al. 2002).

For these results to be validated, further repetition with increased number of samples is required. In addition, to complete the information, we need to study the impact of insulin and LH in non-PCOS and PCOS adipocyte testosterone

secretion at a post-transcriptional level. The next section will study testosterone secretion by the ELISA technique.

The experiments were done on *in vitro* differentiated adipocytes. The treatment concentrations chosen were those used in gonadal steroidogenic studies, particularly Munir et al. (2004).

Addition of insulin treatment ranges in non-PCOS culture showed gradual increases in testosterone secretion. From this observation, we may suggest that insulin impacts testosterone metabolism in non-PCOS mature adipocytes at these treatment levels. We took these observations and investigated a possible pathway in which insulin may be impacting testosterone production, similar to that seen in ovarian androgen synthesis.

The protocol used was similar to that used by Munir et al. (2004). They used a PI3Kase inhibitor (LY294002) in combination with insulin treatment concentrations, which had previously been shown to increase testosterone production from adipocytes. The results showed no changes happened between inhibited and untreated culture. It is possible that insulin stimulates testosterone secretion, but not through the PI3Kase pathway. These observations consist with the results of mRNA of target genes.

On the other hand, results of PCOS show that there is no impact of insulin on testosterone secretion. This may indicate insulin resistance or a need for an increase in the concentration of insulin. In addition, when the basal level of testosterone is compared between non-PCOS and PCOS adipocytes, we observed that the level of testosterone secretion by PCOS adipocytes is significantly increased compared to non-PCOS adipocytes. This suggests the intracrine function of adipocytes, and the possibility of the presence of other modulators that regulate androgen production from adipocytes.

In addition, we tested the impact of LH and insulin combined and found that LH has a significant effect on testosterone secretion from non-PCOS adipocytes. In combination with insulin, we observed that there is a change in testosterone production compared with the effect of LH only. However, in PCOS, LH alone or in combination with insulin has no effect. This could

indicate the change in the sensitivity to LH or the need for a high concentration of LH to produce an impact in PCOS.

It appears that adipocytes are able to synthesise androgen, as we confirmed the expression by adipocytes of two main enzymes in androgen metabolism. There is also a possible role of adipocytes in the hyperandrogenemia in PCOS, with lack of insulin and LH effect on the steroidogenesis in adipocytes of PCOS women.

In addition, it appears that the presence of another factor or factors may have a critical role in the development of excess androgen. This is independent of insulin and produced by adipocytes; especially after detection of adipose tissue as an endocrine, intracrine, and paracrine tissue, due to its products - namely adipokines.

These observations suggest that one of the pathologies of PCOS development is due to a dysregulation of the main enzymes in adipocyte steroidogenesis - CYP17A1 and AKR1C3. However, the basis of the dysregulation in the steroidogenesis is unclear. In addition, abnormal regulation of steroidogenesis in PCOS might be due to the high level of LH, or decreased sensitivity to LH.

In summary, by studying the hormonal influences of Insulin and LH on CYP17A1 and AKR1C3 expression levels in non-PCOS and PCOS adipocytes, we found that: Adipocytes are able to synthesise their own steroid hormone, as they express the key enzyme the steroidogenic CYP17A1; the study confirms the role of adipocytes in the activation of inactive steroid hormone (androstenedione) to active form through confirming the expression of AKR1C3 mRNA in adipocytes. In PCOS, it may be suggested that adipocytes have a significant role in the development of high androgen, as the study observed an abundant level of mRNA for CYP17A1 and AKR1C3 in adipocytes in PCOS. Moreover, the basal level of testosterone collected from the media of PCOS is significantly higher than that of non-PCOS.

Insulin has no significant impact in the regulation of CYP17A1 expression in normal and PCOS adipocytes. This may be explained as following the presence of modulator or modulators that regulate CYP17A1 at expression-level independent of insulin. The other explanation is that a genetic defect is responsible for the dysregulation of CYP17A1 in PCOS women.

Regarding AKR1C3 mRNA expression, in non-PCOS cases insulin at high concentration enhances AKR1C3 expression. However, in PCOS cases it has no significant effect on expression. The first interpretation for this observation is that in normal women, the insulin receptor responds normally to insulin - this means there is no insulin resistance. While in PCOS, it may need a higher concentration of insulin or there is genetic effect in the insulin response, with the presence of environmental factors predisposing women with PCOS to develop insulin resistance and compensatory hyperinsulinemia. Alternatively, this may be related to the presence of other regulators that regulate AKR1C3 expression independent of insulin. In addition, we observed that insulin produces its impact on AKR1C3 expression in non-PCOS adipocytes through other signal transduction pathways, most likely MAPK, which needs to be studied.

Regarding the impact of LH, we found that LH in non-PCOS has significant impact, but in PCOS has no effect. This may need high concentrations of LH to produce its impact, or there is a defect in the sensitivity to LH due to downregulation of LH receptors.

CHAPTER 4 DISCUSSION

4.1 General discussion

PCOS is a complex disorder with numerous contributing environmental and genetic factors. Multidisciplinary efforts involving a combination of clinical, biochemical and laboratory approaches appear mandatory to increase knowledge in the pathophysiology of this disease. By probing the transcriptional activity of tissues, the techniques enabling systemic analysis of adipose tissue gene expression have been shown to be suitable for recognising genes (Kim 2010) and regulatory networks included in human adipose tissue related disorders, such as obesity (Morine, Tierney et al. 2011). Furthermore, mRNA molecules are easily amplified. Therefore, mRNA profiling remains one of the most powerful methods for comprehensive exploration of minute amounts of tissue. For gene expression studies, optimisation of adipose tissue handling and total RNA extraction is an essential step in this study.

Although the main steroidogenic organs in women are ovaries and adrenal glands; peripheral tissue, such as adipose tissue, has shown the ability to produce steroid hormones (Deslypere, Verdonck et al. 1985). Obesity is one of the most important features of PCOS, and occurs in 38-88% of PCOS cases. Moreover, it is associated with imbalance in sex hormone, particularly androgen (Barbieri, Makris et al. 1986).

This association is considered the major factor in androgen excess, and opens the door to further investigations in this field. It is clear that androgens have a critical role in body fat distribution and are related to central obesity. It is understood that increased fat deposition aggravates clinical features of hyperandrogenemia. Soule et al. (1996) showed that increases in the accumulation of abdominal fat was associated with exogenous treatment with androgen in female to male transsexuals.

It is clear that obesity is an important risk factor in the development of PCOS. However, its implication in the pathophysiology of this syndrome is still unclear. Recently, the ability of adipose tissue to manifest endocrine, autocrine and paracrine function was discovered (Mohamed-Ali, Pinkney et al. 1998).

Studying the metabolic and endocrine dysfunction of adipose tissue is of clinical importance to understand the role of adipose tissue in the pathogenesis of PCOS.

Therefore, this study has investigated the androgen metabolism at two levels, at adipose tissue and at cellular levels. It postulates the significance of this tissue in the pathophysiology of PCOS. In this study, we attempted to examine the difference between non-PCOS and PCOS adipocytes, and the association between PCOS adipocytes and hyperandrogenemia.

The significance of androgen synthesis has been seen throughout the study of PCOS and ovarian biosynthesis, and has been found to account for 50% of production in non-PCOS women - this percentage was found to be critically increased in PCOS women.

Even though the mechanism is still not determined, multiple enzymes and signalling pathways have been investigated. CYP17A1 (17α-hydroxylase/ 17, 20 lyase) has been proposed to be included, as it has a key role in androgen biosynthesis. This enzyme has been reported to respond positively to the impact of insulin as well as LH, which are elevated in women with PCOS, within ovarian androgen biosynthesis (Gilling-Smith, Willis et al. 1994).

The main findings of this research are: the extraction of total RNA from adipose tissue of women with PCOS may be more difficult than from non-PCOS. Regarding adipose tissue and steroidogenesis, androgen biosynthesis in adipose tissue has the ability to produce its own sex hormone, as it expresses the CYP17A1 enzyme, which is the key enzyme in steroid hormone. Also, it expresses the androgen regulatory gene; AKR1C3 mRNA is significantly higher than in -PCOS adipose tissue. Based on this it postulates that adipose tissue may have an important role in the development of excess androgen in women with PCOS.

In addition, we found difficulties in the optimisation of adipocyte culture in terms of maintenance. The study has shown the difference in the rate of differentiation between PCOS and non-PCOS adipocytes. PCOS adipocytes were shown to have a decreased rate of differentiation in comparison with non-PCOS adipocytes.

Furthermore, we detected changes in the hormonal response of adipocytes; this response includes gene expression and testosterone secretion. Insulin has no impact on CYP17A1 mRNA expression in both non-PCOS and PCOS adipocytes, as well as AKR1C3 mRNA expression in PCOS adipocytes. However, in non-PCOS adipocytes at high dose, it has a significant effect on AKR1C3 mRNA expression. Insulin produces its effect on the expression of AKR1C3 in non-PCOS adipocytes independently of the PI3Kase signalling pathway. Interestingly LH alone has an impact on the expression of CYP17A1 and AKR1C3 mRNA only on non-PCOS adipocytes, and has no influence on the androgen metabolic enzymes in combination with insulin. Secretion of testosterone from non-PCOS and PCOS adipocytes *in vitro.* PCOS adipocytes were seen to have increased testosterone production when compared with non-PCOS adipocytes. With insulin treatment, testosterone secretion from non-PCOS adipocytes was increased, whereas PCOS adipocytes remained unaffected by insulin stimulation and produced constant levels of testosterone. The molecular mechanism mediating adipocyte testosterone secretion in response to insulin treatment in non- PCOS requires further investigation. Testosterone secretion from non-PCOS adipocytes responds to LH treatment and is unaffected in PCOS adipocytes. LH in combination with insulin has no impact on testosterone secretion in both non-PCOS and PCOS adipocytes.

With the prevalence of high levels of LH in women with PCOS, we investigated the possibility of an association with adipocyte metabolism including sex hormone and the insulin pathway. In fact, in this area of investigation, the relationship between LH and adipocyte regulation is still not fully understood; but indicates LH impacting adipocytes metabolism. Our results found PCOS adipocytes failed to respond to LH; this postulates that a dysfunction may be present in PCOS adipocytes, which removes the possible effect of LH on the steroid hormone metabolism, downregulation of LH receptors, factor or factors in the adipocytes environment impacts on its effect on adipocytes hormone metabolism; here, further research and study is required.

As mentioned before, the most important feature of PCOS is hyperandrogenism, obesity with features of metabolic syndrome such as insulin resistance, and diabetes (Sharpless 2003). The main cause of PCOS is still vague, and it is suspected that obesity may play a role in the pathophysiology of this syndrome (Barber, McCarthy et al. 2006). It is believed that obesity may be related to insulin resistance and compensatory hyperinsulinemia due to increases in adipose tissue production of fatty acids, glycerol, hormones, and pro-inflammatory cytokines (Gambineri, Pelusi et al. 2002). A subsequent relationship is suggested to have an important involvement in the hyperandrogenemia features of the syndrome. In addition, there are studies on the association between adipose tissue and androgen, and suspect that increases in the activity of the hypothalamic pituitary adrenal axis (Soule et al., 1996) - and decreases in the SHBG production and high dietary lipid intake (Preziosi, Barrett-Connor et al. 1993), may enhance high androgen synthesis.

These findings have shown evidence of androgen synthesis and production not only through the presence of testosterone within the culture media, but also through the detection the key enzymes CYP17A1 and AKR1C3. These are involved in androgen synthesis, where the level of their expression in PCOS adipose tissue is higher than in non-PCOS adipose tissue. Similarly, testosterone levels in the culture media of PCOS adipocytes are higher than those in the culture media of non-PCOS adipocytes.

These findings may suggest not only the presence of androgen processing, but also a biosynthesis pathway. If this were the case, it will produce avenues for investigation into the further influx of androgen possibly associated with PCOS - and open an area which may be involved in hyperproduction of androgen. These results suggest localised androgen synthesis and the possibility of involvement of adipose tissue in development of excess androgen.

Androgen biosynthesis in the main androgen synthesis organs - ovarian thecal cells and adrenal cells - increase in PCOS patients. This increase is in response to high insulin levels (Rojas, Chavez et al. 2014). In addition, Munir et al. (2004) observed that the key enzyme for androgen synthesis in ovarian thecal

cells is significantly increased in response to ranges of insulin and LH treatments.

4.2 Future work

A number of limitations and future directions need to be addressed regarding the current work:

Firstly, the studies undertaken did not examine the activity rate of the main androgen metabolic enzyme, CYP17A1 and AKR1C3 (17β-HSD) in adipose tissue of women with PCOS and compared with non-PCOS, due to time limitation. The majority of previous studies have detected the activity of these enzymes in non-PCOS adipose tissue. Further work could also include investigation or functional analysis of these two enzymes in adipose tissue of PCOS women.

As we studied CYP17A1, AKR1C3 at mRNA level which may not always reflect the protein amount (Greenbaum 2003). Also, mRNA levels only partly correlate with protein concentration. Therefore, studying protein amount is considered as a reliable indicator of functioning enzyme. In order to support our results at mRNA level we studied AKR1C3 protein expression by WB technique; however, our results need validation by repeating the experiment (WB) with increased sample numbers. Furthermore, in future studies it would be interesting to look at the changes in CYP17A1 and AKR1C3 protein expression, and to accurately quantify protein; further study using immunofluorescent staining of protein would allow quantification of protein in PCOS and compared with non PCOS.

Secondly, we showed that impact of insulin on AKR1C3 in non-PCOS adipocytes is dose dependant. In order to understand the underlying mechanism of insulin in androgen metabolism in adipose tissue, we investigated its cellular mechanism by blocking the PI3Kase signalling pathway using a PI3K inhibitor. We did not examine the other signal pathway of insulin, MAPK, a mitogenic pathway which is also activated by insulin through the binding of phosphorylated IRS or Shc with Grb-2/SOS complexes, leading to MAPK activation through p21Ras and Raf-1 (Fröjdö 2009). Resulting ignore this important pathway of insulin function which may have a role in insulin action

on androgen metabolism. Hence, it is important to investigate the other signal of insulin pathway, by application of a MAPK inhibitor or agonist.

Thirdly, physiological concentrations (10nm/l) of LH were used in this study, but due to time limitations and small sample numbers it was hard to investigate LH impact at a wide range of concentrations. Hence, it also would be interesting to assess the effect of LH on androgen metabolism in adipocytes with increasing ranges of LH levels. Particularly, based on previous studies which observed that continuous elevation and stimulation of LH would translate into augmented synthesis of ovarian steroid hormones, particularly androgens (Kim 2005).

Fourth, we showed that basal levels of CYP17A1 and AKR1C3 mRNA are abundant in adipocytes of women with PCOS; also we found the basal level of testosterone secretion in PCOS was significantly greater than that of non-PCOS. Based on these findings and on the literature review suggest that involving of local factor/factors work as regulator in androgen metabolism in PCOS. It can be suggested that the adipocyte products, adipokines, through their autocrine, paracrine, and intracrine function, may link between adipose tissue and PCOS development - through their impact on sex steroid secretion and the metabolism of adipocytes. Munir and colleagues observed that the mean level of serum resistin in 45 women with PCOS was raised compared with 74 control women (Munir, Yen et al. 2005). Furthermore, it appears that abnormal production of adipokines in PCOS is implicated in the development of hyperandrogenaemia, which is the most consistent feature of this disorder, in addition to its involvement in the metabolic dysfunction (Baber, McCarthy 2006). In support to these, a study on bovine ovarian cell culture showed the inhibitory direct effect of adiponectin on theca cell steroidogenesis; adiponectin decreased insulin-induced progesterone and androstenedione production, and reduced IGF-1 induced gene expression from CYP17A1, and LH receptors in theca cells (Lagaly, Aad 2008). The current data from the literature about adipokine cytokines in PCOS, and their mechanism in the metabolism of sex

steroid in adipose tissue, are still not conclusive. In future, it would be interesting to study this modulator/modulators and their relationship with insulin in androgen metabolism in the adipose tissue of women with PCOS. Also, future work should include studies on the potential effects of insulin on adipokine and cytokine products of adipose tissue, to determine their role in the metabolism of androgens. By studying the impact of insulin on the secretion of adipokines and cytokines, it will be a novel area of future research would help to explore and to understand the underlying mechanism of insulin in androgen metabolism in adipose tissue.

The results of our study have highlighted the need to understand the modulators, which may have a role in the dysregulation of androgen metabolism in adipose tissue of women with PCOS. This would pave the way to understanding the underlying mechanism through which adipose tissue is involved in the PCOS development. I have demonstrated a study performed by an undergraduate student in the laboratory of Nottingham University. This studied the differences in the expression of mRNA levels of IL6 between non-PCOS and PCOS adipocytes, and examined the impact of different concentrations of insulin on the expression of cytokines (TNF-α and IL6) on SAT. The results were important and interesting, as they have shown high statistically significant values between the non-PCOS and PCOS subgroups of both cytokines, as well as high values of mRNA expression of IL-6 in the PCOS subgroup.

Further studies are needed to identify an important connection between androgen excess, insulin resistance, and adipose tissue, by studying adipocytes products in PCOS and the impact of hormones on the expression of these products.

Finally, with regard to the controversial considerations about the management of PCOS syndrome, further studies are needed to reveal the intricacies within the pathophysiology of PCOS and the correct relationships between insulin and androgens; as well as other important hormonal disturbances, particularly changes within steroid hormone metabolism, adipokines and cytokines. This would enable the formulation of improved

approaches to the treatment of obesity and IR as therapeutic aims in women with PCOS; in order to improve their quality of life and especially their fertility outcomes, which are often the main concern of both patients and physicians in the management of this pathology.

4.3 Conclusion

The series of studies described in this thesis demonstrated the significance of adipose tissue as a source of androgen, and that it plays a crucial role in the pathology of PCOS. Although the published data are limited, this research describes for the first time the presence and modulation of androgen biosynthesis at a transcriptional and protein level in PCOS adipose tissue. This work also explored the impact of insulin and LH on adipocyte steroid synthesis at cellular level in *in vitro* PCOS adipocytes. So with the findings of this research, we highlighted a new pathway in adipocyte function and dysfunction, thus increasing our understanding of the pathophysiology of PCOS, in order to help women with this complicated and vague condition.

Although the preferred treatment for PCOS is still controversial, our increasing understanding of the mechanism of development of PCOS, and the linkage between adipose tissue and reproductive problems will dramatically enhance and change the treatment approaches for reproductive endocrinologists. A great opportunity to identify the treatment for PCOS in the future is based on the therapeutic manipulation of adipocytes. A focused approach towards a more complete understanding of the physiology in women with PCOS is both timely and necessary.

APPENDIX

Reagents and solution composition

RNase ZAP® Sigma (R2020) – 250mL

Chloroform

Isopropanol

Ethanol 75%

TRI Reagent® Sigma (T2924) – 100mL

RNase-Free Water

RNase Inhibitor (Applied Biosystems) (20 U/µL) - 100021540

10X RT Random Primers (Applied Biosystems) 4319979

dNTP Mix (Applied Biosystems) 100mM 362271

Multi Scribe Reverse Transcriptase (Applied Biosystems) 50 units/µL 4308228

10X RT Buffer (Applied Biosystems) 4319981

10X Tris-Borate (10X TBE) electrophoresis buffer

Ultrapure™ Agarose powder (Invitrogen)

1Kb DNA Ladder

Ethidium Bromide

Loading Buffer

PBS (Phosphate Buffered Saline)

Wash Buffer

Reagent Diluent

Substrate solution

Stop Solution

PBA working dilution:

200ml PBD stock, 300ml 5 M Na Cl, 770ml H 2 O

PBA stock:

Na2 HPO4, NaH 2PO4, PH 7.2-7.4 with NaOH.

10% Ammonium persulphate (AP):

0.1g Ammonium persulphate in 1 ml H_2O

4% W/V Paraformaldehyde:

4g PFA dissolved in d H_2O at 60°C. PH 7.4 using 1M NaOH. Stored at -80°C for 3 months.

HBSS:

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

Homogenisation buffer (adipose tissue):

320m M Sucrose, 10m M Tris base p H 7.8, 50m M KCl, 1m M ethylenediaminetetraacetic acid (EDTA), 0.5% Igebal, protease inhibitor cocktail (Sigma)(1:500) supplemented with fresh phosphatase inhibitor cocktail (Sigma) 1:10

Enzyme dissociation solution:

Collagenase I, 2mg/ml, hyaluronidase 0.5 mg/ml and DNase I 15 Kunits

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 KC, 25NaHCO3, 2.5MgSO4, 1.6 CaCl2, 1.2 KH2PO4, 5.5 glucose, 0.034 EDETA p H 7.4 stored at 4 °C.

Resolving gel:

12%: 4.1ml d H2O2, 3.3 ml Acrylamide, 2.5ml resolving gel buffer (Tris base p H8.8), 100µl 10% SDS, 100µl ammonium persulfate (APS), 4µl N,N,N' N'-Tetramethlyenediamine (TEMED)

Running buffer:

15.1g Tris, 94g Glycine, 50ml 10% SDS in 1 LH2O

Solubilisation buffer:

20m M Tris base p H 7.5, 10m M EDTA, 120m M NaCl, 50m M KCL, 2.5% I gebal supplemented with fresh protease inhibitor cocktail (Sigma)(1:500) and phosphatase inhibitor cocktail (Sigma) 1:100.

Staking gel:

4%: 3.4ml d H2O2, 0.85 ml Acrylamide, 0.625 ml resolving gel buffer (Tris base p H8.8), 50µl 10% SDS, 50µl TEMED.

TBS-Tween (TBST) 0.1%:

1 L TBS 1ml Tween-20

Transfer buffer:

24m M Tris base, 80m M glycine, 20% methanol.

Tris buffered saline (TBS):

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

Working dilutions of antibodies

Antibody	Host	Type	Secondary antibody& conjugate
AKR1C3		Polyclonal	1:1000
Beta actin DakoCytomation)	Rabbit	Polyclonal	1:30000 AP (Sigma)

Laboratory equipment

BIOHIT PROLINE Pipette (100-1000 μL)

BIOHIT PROLINE Pipette (20-200 μL)

BIOHIT PROLINE Pipette (0.1-2.5 μL)

Sterile Pipette Tips (100-1000μL) – Alpha Laboratories ZAP Premier Ext Tip

Sterile Pipette Tips (1-200 μL) – Alpha Laboratories ZAP Premier Ext Tip

Sterile Pipette Tips (0.1-10 μL) – Alpha Laboratories ZAP Premier Ext Tip

Sterile disposable forceps (Servoprax®) –HF 301

Homogeniser (Janke & Kunkel) IKA® Labortechnik UHra - turrax T25

500 ml Erlenmeyer flask

Electrophoresis horizontal gel box with comb

Electrophoresis - Power supply

Electrophoresis – UV light box with camera

Microwave

Gloves

1 mL microcentrifuge tubes

Dissecting scalpels

Scales

Polystyrene bucket

Petri dish

Plastic vial

Plastic rack

Suppliers

Supplier	Contact Information
Abcam	330 Cambridge Science Cambridge CB4 OFL UK Web: http://www.abcam.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
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