Unravelling the link between insulin resistance and androgen excess

by

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Abstract

Insulin resistance and androgen excess are the cardinal phenotypic features of polycystic ovary syndrome (PCOS). The severity of hyperandrogenism and metabolic dysfunction in PCOS are closely correlated. Aldoketoreductase type 1C3 (AKR1C3) is an important source of androgen generation in human adipose tissue, and may represent a link between androgen metabolism and metabolic disease in PCOS. We performed integrated *in vitro* studies using a human preadipocyte cell line and primary cultures of human adipocytes, coupled with *in vivo* deep phenotyping of PCOS women and age- and BMI-matched controls.

We have shown that insulin upregulates AKR1C3 activity in primary female subcutaneous adipocytes. AKR1C3 mRNA expression increased with obesity. Androgens were found to increase lipid accumulation in human adipocytes. In clinical studies, androgen exposure induced relative suppression of adipose lipolysis in PCOS women, supporting a role for androgens in lipid accumulation. Androgens were detectable in adipose fluid from PCOS women, and correlated with systemic markers of androgen metabolism.

Using comprehensive *in vivo*, *ex vivo*, and *in vitro* techniques, we have shown regulation of adipose androgen generation through AKR1C3, with evidence of a vicious circle of hyperinsulinaemia, adipose androgen generation and lipid accumulation. These data identify AKR1C3 as a promising therapeutic target in PCOS.

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Abbreviations

11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
17OHP	17-hydroxyprogesterone
17β-HSD	17β-hydroxysteroid dehydrogenase
21OHD	21-hydroxylase deficiency
3α-HSD	3α-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
5α-R	5α-reductase
A	androstenedione
ACC1	acetyl coA carboxylase type 1
ACC2	acetyl coA carboxylase type 2
ACS	acyl coA synthetase
ACTH	adrenocorticotrophic hormone
AE	androgen excess
AI	aromatase inhibitor
AKR1C1	aldoketoreductase type 1 C1
AKR1C2	aldoketoreductase type 1 C3
AKR1C3	aldoketoreductase type 1 C3
AMPK	5'-adenosine monophosphate-activated protein kinase
An	androsterone
Anov	anovulation
AR	androgen receptor
ARE	androgen response elements
ARKO	androgen receptor knock-out
ASC	acyl CoA synthase
ATGL	adipose triglyceride lipase
ATP	adenosine triphosphate
AU	arbitrary units
AUC	area under the curve
BAT	brown adipose tissue
BMI	body mass index
BP	blood pressure
BWH	Birmingham Women's Hospital
C/EBP	CAAT/Enhancer-binding protein
CAH	congenital adrenal hyperplasia
cDNA	complementary deoxyribonucleic acid
CGL	congenital generalised lipodystrophy
CI	confidence interval
CL	citrate lyase
CNS	central nervous system
CPP	central precocious puberty
CPT1	carnitine palmitoyl transferase I
CRH	corticotropin-releasing hormone
CT	computerised tomography
CVD	cardiovascular disease
CYP19A1	placental aromatase
DAG	diacylglycerol
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone
DMEM	dulbecco's modified eagles medium
DNA	deoxyribonucleic acid
DNL	*de novo* lipogenesis
DXA	dual x-ray absorptiometry
E	cortisone
E1	oestrone
E2	oestradiol

E3	oestriol
ECM	extracellular matrix
Et	etiocholanolone
F	cortisol
FABP4	fatty acid binding protein-4
FAI	free androgen index
FAS	fatty acid synthase
FCS	fetal calf serum
FFA	free fatty acid
FPL	familial partial lipodystrophy
FSH	follicle-stimulating hormone
G3PDH	glycerol-3-phosphate dehydrogenase
GC	glucocorticoid
GC/MS	gas chromatography mass spectrometry
GLUT	glucose transporter
GnRH	gonadotrophin-releasing hormone
GPAT	glycerol-3-phosphate acyl transferase
GR	glucocorticoid receptor
HA	high androstenedione
HAIR-AN	hyperandrogenism-insulin resistance-acanthosis nigricans
HDL	high-density lipoprotein
hMSC	human mesenchymal stem cell
HMW	high molecular weight
HOMA-IR	homeostatic model assessment of insulin resistance
HPA	hypothalamic-pituitary adrenal
HPG	hypothalamic-pituitary-gonadal
HRT	hormone replacement therapy
HSL	hormone sensitive lipase
HT	high testosterone
IGF-1	insulin-like growth factor-1
INSR	insulin receptor
IR	insulin resistance
IRS	insulin receptor substrate
ISI	insulin sensitivity index
kDa	kilodalton
KLF15	kruppel-like factor 15
KRB	Krebs Ringer bicarbonate
LAP	Lipid accumulation product
LC-MS/MS	liquid chromatography tandem mass spectrometry
LDL	low-density lipoprotein
LH	luteinising hormone
LMW	low molecular weight
LPL	lipoprotein lipase
MAG	monoacylglycerol
MAPK	mitogen-activated protein kinase
MGL	monoglyceride lipase
MGL	monoglyceride lipase
MR	mineralocorticoid receptor
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MTBE	tert-butyl-methyl-ether
NA	normal androstenedione
NADH	nicotinamide adenine dinucleotide
NAFLD	non-alcoholic fatty liver disease
NEFA	non-esterified fatty acids
NHP	non-human primate
NIH	National Institutes of Health
NSAID	non-steroidal anti-inflammatory drug
NT	normal testosterone
OGTT	oral glucose tolerance test
OM	omental

PA	premature adrenarche
PBS	phosphate buffered saline
PCO	polycystic ovaries
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKB/akt	protein kinase B/akt
PKC	protein kinase C
PP	premature pubarche
PPARγ	peroxisome proliferator-activated receptor gamma
Pref-1	preadipocyte factor-1
QEHB	Queen Elizabeth Hospital Birmingham
REC	research ethics committee
RIA	radioimmunoassay
RMS	Rabson-Mendenhall syndrome
RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
SC	subcutaneous
SD	standard deviation
SEM	standard error of the mean
SERM	selective estrogen receptor modulator
SGBS	Simpson-Golabi-Behmel syndrome
SHBG	sex hormone-binding globulin
SIR	severe insulin resistance
SPSS	Statistical Package for the Social Sciences
SRD5A1	5α-reductase type 1
SRD5A2	5α-reductase type 2
SREBP	sterol-regulatory element binding protein
StAR	steroidogenesis acute regulatory protein
STS	steroid sulfatase deficiency
SULT	sulfotransferase
T	testosterone
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
T3	tri-iodothyronine
TAG	triacylglycerol
TART	testicular adrenal rest tumour
TChol	total cholesterol
TG	triglyceride
THE	tetrahydrocortisone
THF	tetrahydrocortisol
TSH	thyroid-stimulating hormone
UFE	urinary free cortisone
UFF	urinary free cortisol
UHB	University Hospitals Birmingham
VAI	visceral adiposity index
VLDL	very-low density lipoprotein
WAT	white adipose tissue
WHR	waist:hip ratio
WTCRF	Wellcome Trust Clinical Research Facility
$ZnSO_4$	zinc sulphate
Δct	delta cycle threshold

1 General Introduction

Parts of this chapter have been published as:

Understanding androgen action in adipose tissue

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1.1 Introduction

Polycystic ovary syndrome (PCOS) is a clinical triad of anovulation, insulin resistance (IR) and androgen excess, with an estimated prevalence of 5-10% in women of reproductive age (Franks, 1995, Dunaif, 1997). Androgen excess (or hyperandrogenism), defined as clinical or biochemical evidence of elevated androgens in females, generally presents clinically with male-pattern hair growth (hirsutism) or acne (Housman and Reynolds, 2014), Figure 1-1.

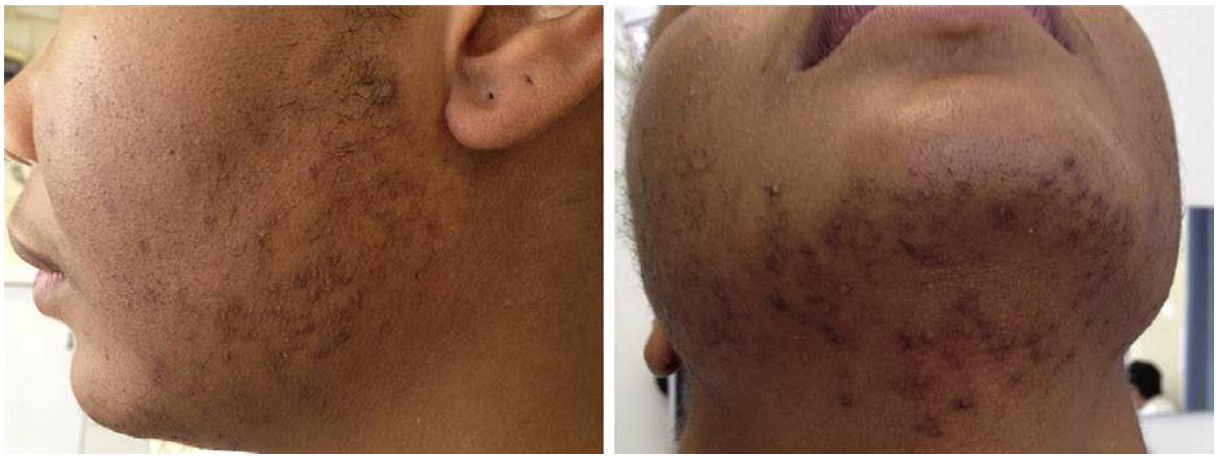

Figure 1-1 Clinical hyperandrogenism: hirsutism and acne in a woman with PCOS (Housman and Reynolds, 2014). Reproduced with permission from Elsevier.

Women with PCOS are predisposed to glucose intolerance and metabolic syndrome (Taponen et al., 2004), with associated risk factors for cardiovascular disease and a likely increased risk in future cardiovascular events in the postmenopausal period (Mani et al., 2013) (Figure 1-2). Anovulatory infertility secondary to PCOS is the most common cause of subfertility in the developed world, and consumes the majority of healthcare budgets allocated to assisted conception (Fauser et al., 2012). Insulin resistance and androgen excess are the cardinal features driving the clinical phenotype in PCOS, but the precise interplay between

androgen metabolism and insulin sensitivity is poorly understood; hyperinsulinaemia leads to androgen excess in females of reproductive age, while women with monogenic disorders of androgen excess develop a PCOS-like phenotype with polycystic ovaries and insulin resistance.

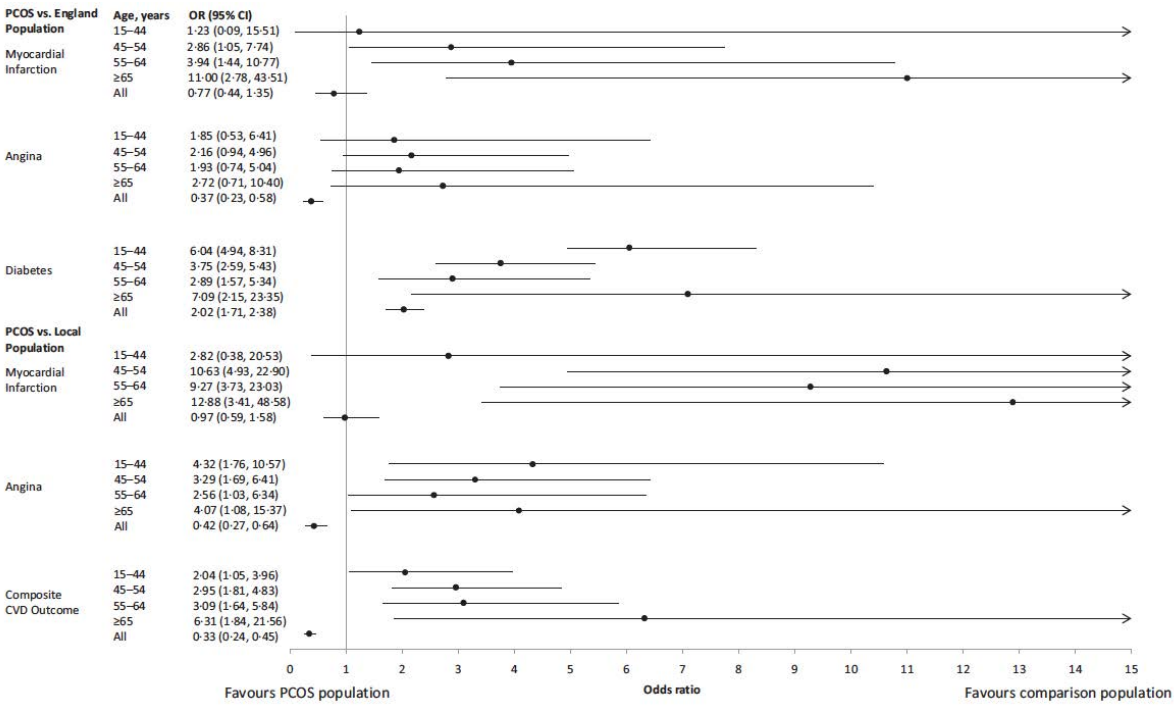

Figure 1-2 Odds ratio of cardiovascular events and T2DM in POCS vs local or national female population. Age-specific prevalence of MI, angina and composite CV outcome was significantly higher in PCOS women over 45 years old compared to the local female population (Mani et al., 2013). Reproduced with permission from Wiley and Sons.

PCOS has traditionally been regarded as a disorder of primary ovarian, and to a lesser extent, adrenal, origin (Franks et al., 1999). However, this dogma has recently been challenged, leading to a paradigm shift in our understanding of the origins of androgen excess and metabolic dysfunction in PCOS. Increased focus on extra-ovarian and extra-adrenal androgen activation has highlighted the role of peripheral tissues such as adipose in local

androgen generation (Fassnacht et al., 2003, Quinkler et al., 2004). Recent studies have explored how systemic upregulation of crucial androgen-activating enzymes such as 5α-reductase (5αR) and aldoketoreductase type 1 C3 (AKR1C3) may contribute to androgen excess in PCOS (Stewart et al., 1990), and how this process may be driven by insulin (Vassiliadi et al., 2009). Our understanding of the role of androgens in adipose tissue is also evolving; adipose tissue is capable of local androgen activation and inactivation (Blouin et al., 2009a), while androgens themselves modulate adipose tissue function, lipid metabolism and insulin sensitivity (Corbould, 2007). Similar to effects observed with glucocorticoids, regulation by androgens of the balance between lipid accumulation and lipid breakdown ('lipid flux') in adipose tissue may crucially orchestrate adverse androgen-mediated effects on metabolism and insulin sensitivity (Gathercole et al., 2011).

1.2 An introduction to polycystic ovary syndrome (PCOS)

PCOS is the most common endocrine disorder, affecting up to 10% of women of reproductive age. The clinical syndrome was initially described by Stein and Leventhal in 1935, although polycystic ovaries (cystic oophoritis, sclerocystic ovaries) associated with androgen excess and infertility were noted as far back as 1721. Diagnosing a 'syndrome', traditionally defined by a collection of signs, symptoms and clinical features, has proven exceptionally difficult, however.

1.2.1 Diagnostic criteria

There have been numerous attempts over the last two decades to reach consensus on diagnostic criteria for PCOS (Table 1-1) (Fauser et al., 2012, Rotterdam, 2004b, Chang and

Katz, 1999). Chronic anovulation and clinical and/or biochemical hyperandrogenism remain the key clinical components of all proposed diagnostic criteria. The addition of sonographic features of polycystic ovaries (PCO) to the broadened 2003 Rotterdam criteria (sponsored by the European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine) encompasses those patients who may have PCO on ultrasound with co-existent androgen excess but without ovulatory dysfunction, or anovulation without hyperandrogenism. It was increasingly recognized that this subgroup of women, who did not meet diagnostic criteria for PCOS according to the initial 1990 National Institute of Health (NIH) guidelines, had convincing clinical evidence of the disorder and should be classified as such accordingly (Carmina and Lobo, 2001).

Table 1-1 1990 (NIH) and 2003 (Rotterdam) criteria for diagnosis of PCOS

Revised diagnostic criteria of polycystic ovary syndrome
1990 Criteria (both criteria required) • Chronic anovulation and • Clinical and/or biochemical signs of hyperandrogenism and exclusion of other aetiologies
Revised 2003 criteria (2 out of 3 required) (Rotterdam, 2004a, Rotterdam, 2004b) • Oligo- or anovulation • Clinical and/or biochemical signs of hyperandrogenism • Polycystic ovaries on ultrasound *and* exclusion of other aetiologies (congenital adrenal hyperplasia, androgen-secreting tumours, primary disorders of insulin resistance, Cushing's syndrome)

Application of the Rotterdam criteria has been controversial, and resulted in a significant increase in the population deemed to be affected (Goodarzi and Azziz, 2006, Barber et al., 2007). Four phenotypic subgroups may now be defined by the presence of the following: 1) androgen excess, anovulation and PCO (AE, Anov + PCO), 2) Anov + PCO, 3) AE + Anov and 4) AE + PCO. The majority of women with PCOS appear to fall into the first

diagnostic category (Welt et al., 2006). It has been suggested that women with non-hyperandrogenic PCOS have a metabolic profile similar to that of healthy controls (Barber et al., 2007). This assumption has recently been disputed (O'Reilly et al., 2014b), with biochemical evidence of insulin resistance found even in non-hyperandrogenic women in one study. However, the same study showed lower insulin sensitivity in those women with androgen excess than without, and it is increasingly clear that circulating androgen burden correlates inversely with insulin sensitivity in PCOS (Pugeat et al., 2000, Legro et al., 2002).

A third task force attempted to further explore the diagnostic conundrum of PCOS. The Androgen Excess Society (AES) recognized four cardinal features of PCOS: 1) ovulatory and menstrual dysfunction, 2) biochemical hyperandrogenaemia, 3) clinical hyperandrogenism and 4) polycystic ovaries (Azziz et al., 2006). Similar to previously published criteria, the contributors concluded that insulin resistance and metabolic derangements, whilst prevalent in a significant proportion of patients, were not an essential component for diagnosis of the syndrome. However, hyperandrogenism was considered a mandatory component of the syndrome, and women with ovulatory dysfunction and PCOS (Anov + PCO) without androgen excess were therefore not considered to have PCOS according to this categorization.

1. Hyperandrogenism: hirsutism and/or hyperandrogenaemia

and

2. Ovarian dysfunction: Oligo-ovulation and/or polycystic ovaries

and

3. Exclusion of other androgen excess or related disorders.

A recent publication has reported that the prevalence rates of PCOS in a large predominantly Caucasian population according to NIH, Rotterdam and AE-PCOS Society criteria are 6.1, 19.9 and 15.3%, respectively (Yildiz et al., 2012).

1.2.2 Clinical features

It is important to recognize that no single diagnostic criterion is sufficient for a diagnosis of PCOS. The presence of insulin resistance, metabolic dysfunction or obesity is not required to diagnose PCOS according to currently accepted criteria. This notwithstanding, insulin resistance in women with PCOS is present in 50-65% of cases, in both obese and nonobese subjects (Dunaif et al., 1989). As is clear from long-standing debate on diagnostic criteria, PCOS has a distinctly heterogeneous clinical phenotype, leading many authors to speculate that it may represent the common clinical consequence of a large range of diverse disorders of androgen metabolism and insulin sensitivity, the so-called ‘fruit bowl’ concept. PCOS is associated clinically with anovulatory infertility, dysfunctional bleeding, androgen excess, insulin resistance, obesity and metabolic syndrome (Dunaif, 1997). Direct longitudinal associations with cardiovascular disease, sleep apnoea and endometrial carcinoma have also been elucidated (Azziz et al., 2005, Morton, 2013, Barry et al., 2014, Mani et al., 2013). However, the vast majority of women with PCOS will present with clinical signs of hyperandrogenism, or anovulation manifest by primary subfertility or oligomenorrhoea (Legro et al., 2014).

1.2.2.1 Anovulation

Polycystic ovary syndrome is the most common cause of anovulatory infertility (Rotterdam, 2004b). Anovulation presents clinically as menstrual irregularity, with either infrequent menses (oligomenorrhoea) or frequent, excessive and painful menstrual bleeding (dysfunctional uterine bleeding) (Franks, 1995). Polycystic ovaries (PCO) are present in most patients with PCOS, although their presence alone is not diagnostic of the clinical and biochemical syndrome. PCO may be detected ultrasonographically in up to 22% of the normal, non-hyperandrogenic population with regular menses (Polson et al., 1988), but the prevalence is much higher in women with hyperandrogenism (Franks, 1989). Increased numbers of antral follicles are characteristic of PCOS; even women with ovulatory PCOS have an increased proportion of follicles arrested in early stages of development (Webber et al., 2003). This finding suggests that abnormal follicle development in PCOS is independent of ovulatory status. The same study found that the density of pre-antral and small antral follicles in the polycystic ovary is six times that of the normal ovary.

Accelerated follicle development with subsequent arrest at the antral or pre-antral stage may be mediated via androgens (Vendola et al., 1999). Numerous pathways have been elucidated, with intra-ovarian androgen excess promoting primordial follicle activation, with subsequent inhibition of early-stage follicle transition leading to follicle arrest (Yang et al., 2010). Increased folliculogenesis in PCOS may lead to suppression of serum follicle-stimulating hormone (FSH), although levels are invariably within the normal range (Laven et al., 2004). Insulin has also been implicated in this process. Severity of anovulation was correlated with insulin resistance and adiposity in a recent study (Androulakis et al., 2014). At a molecular level, insulin has gonadotrophic actions, and may exert synergistic effects with androgens and LH in contributing to arrest of folliculogenesis (Poretsky et al., 1999).

Treatment options for ovulatory dysfunction in PCOS are determined by whether fertility is desirable. In anovulatory women with primary or secondary subfertility who are seeking to conceive, clomiphene citrate is usually first-line therapy when other causes of infertility have been excluded (Legro et al., 2014). This compound is a selective oestrogen receptor modulator (SERM), which antagonizes the negative feedback effect of oestrogen at the level of the hypothalamus, resulting in increased gonadotrophin pulses and increased ovarian stimulation (Thessaloniki, 2008). Metformin may also be used alone or in combination with clomiphene, although there is no evidence that the combination is superior to clomiphene alone (Legro et al., 2007). For oligo- or amenorrhoeic women not seeking fertility, cyclical progestins may be used, for example medroxyprogesterone acetate 10mg daily for 7 days to induce withdrawal bleeding (Rotterdam, 2004b). This can be repeated every three months to prevent endometrial hyperplasia due to amenorrhoea and unopposed oestrogen exposure (Conway et al., 2014). Combined oral contraceptives (COCs) also play a key role in the treatment of menstrual disturbances in PCOS. Typical formulations contain 20-35 µg of ethinylestradiol, with an antiandrogenic progestin such as drospirenone, although concern exists about the adverse cardiovascular and metabolic risk profile of COCs with higher oestrogen doses (Bird et al., 2013, Lidegaard et al., 2011).

1.2.2.2 Hyperandrogenism

Androgen excess may manifest clinically in PCOS women with male-pattern hair growth (hirsutism), acne or frontal alopecia (Franks, 1995). Overt virilisation is rarely associated with the degree of hyperandrogenaemia observed in PCOS. The presence of the latter often suggests underlying severe insulin resistance or androgen-secreting pelvic neoplasms (Azziz et al., 2009). In a large cohort of 950 patients presenting with signs of

clinical hyperandrogenism, over 72% were diagnosed with underlying PCOS (Carmina et al., 2006). Clinical or biochemical hyperandrogenism are crucial diagnostic criteria according to all currently accepted international standards for the diagnosis of PCOS (section 1.2.1).

Hirsutism severity is graded clinically according to the modified Ferriman-Gallwey index (Azziz et al., 1995), whereby 9 areas of the body are assigned a score of 0-4 depending on the density of terminal-end hairs, giving a final score out of 36. Cut-off values are somewhat arbitrary, with wide inter-racial variability, but a value ≥ 8 is generally considered pathological. Hirsutism is found in up to 75% of women with PCOS (DeUgarte et al., 2006). The prevalence of acne in selected cohorts of PCOS women ranges from 12-14% depending on the population studied (Wijeyaratne et al., 2002, Falsetti et al., 2002), while that of androgenic alopecia varies widely between studies, from 5-50%.

Definition of biochemical hyperandrogenism has been controversial. Little consensus has been reached on which androgens should be measured, what constitutes normal reference ranges and which analytical techniques should be employed (O'Reilly et al., 2014b). Serum testosterone (T) measurement has traditionally been the tool-of-choice for assessment of hyperandrogenaemia in PCOS. However, its use is blighted by inaccuracy; T circulates in the low-nanomolar range in females. Equally, circulating testosterone is bound to sex-hormone binding globulin (SHBG), albumin and other proteins, such that measured T is an inaccurate reflection of tissue exposure. SHBG levels fluctuate in the setting of metabolic disturbance, with suppression of hepatic SHBG output by insulin in the setting of hyperinsulinaemia. Measurement of free (unbound) T with equilibrium dialysis or direct radioimmunoassay (RIA) is inaccurate, unreliable and rarely available (Vermeulen et al., 1999). Dehydroepiandrosterone sulphate (DHEAS) circulates in the micromolar range in serum, but unfortunately is elevated in only approximately 25% of patients with PCOS (Orio et al.,

2003). Androstenedione (A) measured by liquid-chromatography tandem mass spectrometry (LC-MS/MS) may be a more reliable marker of PCOS-related androgen excess, circulating at higher concentrations and not influenced by variables such as SHBG (O'Reilly et al., 2014b).

Cosmetic approaches for hirsutism include waxing, threading, shaving, electrolysis and laser therapy. Eflornithine cream inhibits hair growth by irreversibly blocking ornithine decarboxylase, but it typically takes 8 weeks to demonstrate clinical benefit, and should be combined with laser therapy to achieve maximal effect (Ganger and Hamzavi, 2006). COCs may be useful in the treatment of androgen excess, with a suppressive effect on LH-mediated ovarian androgen generation (Conway et al., 2014). Oestrogen-containing preparations have the added bonus of increasing hepatic SHBG production, thereby reducing free (and physiologically active) testosterone levels. Combined preparations containing ethinyoestradiol 20-35 μg and an anti-androgenic progestin such as drospirenone or cyprotereone acetate are used most frequently. Higher oestrogen doses are associated with increased metabolic risk and unfavourable body composition changes (Nader and Diamanti-Kandarakis, 2007, Merz et al., 2006), as well as increasing thromboembolic risk, particularly in formulations containing anti-androgen progestins (Bird et al., 2013). Oral anti-androgen pharmacotherapy can be effective, but the attraction of using such products should be tempered by an awareness of side effect profiles and teratogenic potential. Anti-androgens should only be used with concomitant contraception. Cyproterone acetate may be given in isolation at a dose of 50-100mg day but, like drospirenone, is often combined at lower doses with ethinyloestradiol in an oral contraceptive formulation (Swiglo et al., 2008). Spironolactone, traditionally used as an aldosterone receptor antagonist, may antagonize the androgen receptor, and is effective for hirsutism at doses of 50-200mg/day. Other oral antiandrogen treatments such as the AR

antagonist flutamide (Paradisi and Venturoli, 2010) and the 5αR type 2 inhibitor finasteride (Moghetti et al., 2000) appear to be safe when used with concomitant contraception.

1.2.2.3 Metabolic dysfunction, insulin resistance and cardiovascular disease

Relatively few patients with PCOS present with clinical or biochemical signs of overt metabolic disease such as dyslipidaemia, insulin resistance or type 2 diabetes mellitus (T2DM) as their primary complaint. Nonetheless, the prevalence of insulin resistance is estimated at between 50% and 70% in women with PCOS (Azziz et al., 2009). Dyslipidaemia, usually in the form of low circulating high-density lipoprotein (HDL) cholesterol and elevated triglycerides, as typically found in insulin resistant states, is observed in as many as 70% of patients (Sverrisdottir et al., 2008). Obesity is highly prevalent in PCOS, and appears a crucial regulator of the reduced central and peripheral insulin sensitivity observed in the condition (Escobar-Morreale et al., 2012). Observed metabolic derangements are worse in obese patients, and the progression from insulin resistance to overt T2DM over time is observed predominantly in significantly obese patients (Legro et al., 1999). Obesity therefore appears to be the critical determinant of metabolic disturbance in PCOS, but it is important to emphasise that the degree of insulin resistance is more profound than that observed in body mass index (BMI)-matched obese women without PCOS (Vassiliadi et al., 2009). This suggests a role for other complex and poorly-understood phenomena, including circulating androgen burden, in PCOS-related metabolic disease.

PCOS is associated with a constellation of classic and non-classic cardiovascular risk factors. In addition to an adverse risk profile, young women with PCOS have increased carotid intima-media thickness in multiple studies (Bajuk Studen et al., 2013, Ketel et al., 2010). Hyperandrogenic PCOS women are more likely to develop hepatic

steatosis, another non-classic marker of endogenous cardiovascular risk, independent of BMI or the presence of insulin resistance (Jones et al., 2012). Whether the clustering of risk factors for cardiovascular disease (CVD) observed in PCOS truly translates into increased incidence of or mortality from CVD is contentious, and results between studies have varied widely. One large study found increased cardiovascular mortality in postmenopausal women was associated with longer duration of anovulatory cycles during reproductive years (Shaw et al., 2008). These findings were not replicated in a prospective study over 21 years (Schmidt et al., 2011), but a recent meta-analysis identified women with PCOS at a two-fold increased relative risk for future cardiovascular events and arterial disease, an association not affected after adjustment for BMI (de Groot et al., 2011).

1.3 Physiology of insulin action and insulin resistance

1.3.1 Insulin structure and secretion

Insulin is a crucial regulator of carbohydrate and lipid metabolism in vertebrates, as well as playing a key role in growth and development. It was the first protein to undergo amino acid sequencing, in 1955 by Fred Sanger. The 6000 dalton insulin molecule consists of two polypeptide chains; in humans chain A contains 21 amino acids and chain B has 30 amino acids (Figure 1-3). The chains are tethered by covalent disulfide bonds, while chain A also contains an internal covalent bond between residues A6 to A11 (Yamamoto et al., 2012).

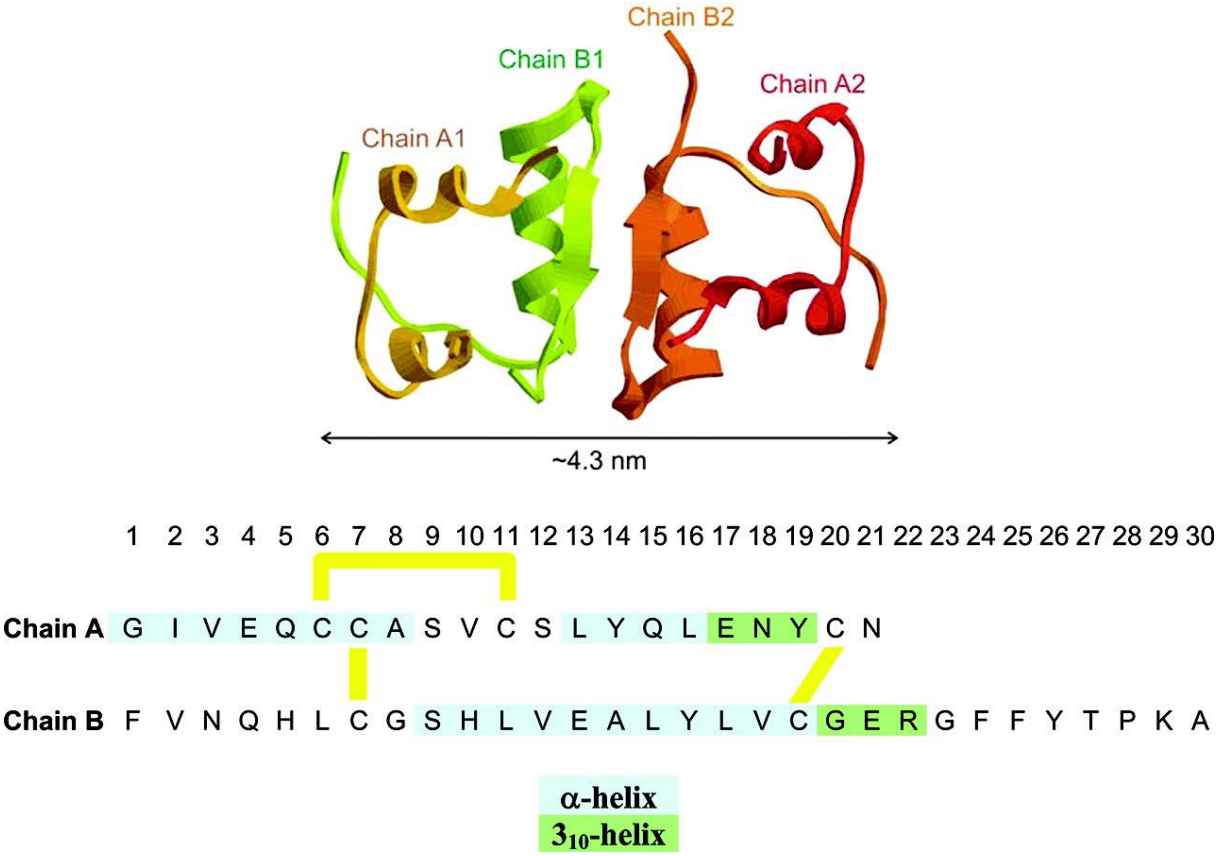

Figure 1-3 Amino acid structure of the human insulin molecule. Chain A contains 21 amino acids; chain B has 30 amino acids. The chains are linked by covalent disulfide bonds. Chain A also has an internal covalent bond between residues A6 and A11 (Yamamoto et al., 2012). Reprinted with kind permission from Yamamoto et al. Copyright 2015 American Chemical Society.

The initial translational product of the insulin gene is preproinsulin, a 'prepeptide' of 24 amino acids which is synthesized in the pancreatic β-cells, located within cellular clusters known as the *islets of Langerhans* (Chan et al., 1976). Preproinsulin is cleaved to proinsulin, which consists of B and A chains linked to a connecting peptide called C-peptide, in the endoplasmic reticulum. Proinsulin is then packaged in storage granules alongside converting proteases (Steiner et al., 1985). These storage granules mature to form secretory granules,

with cleavage of proinsulin to release insulin and C-peptide. Insulin is secreted in response to various physiological and electrophysiological stimuli (Figure 1-4***)***.

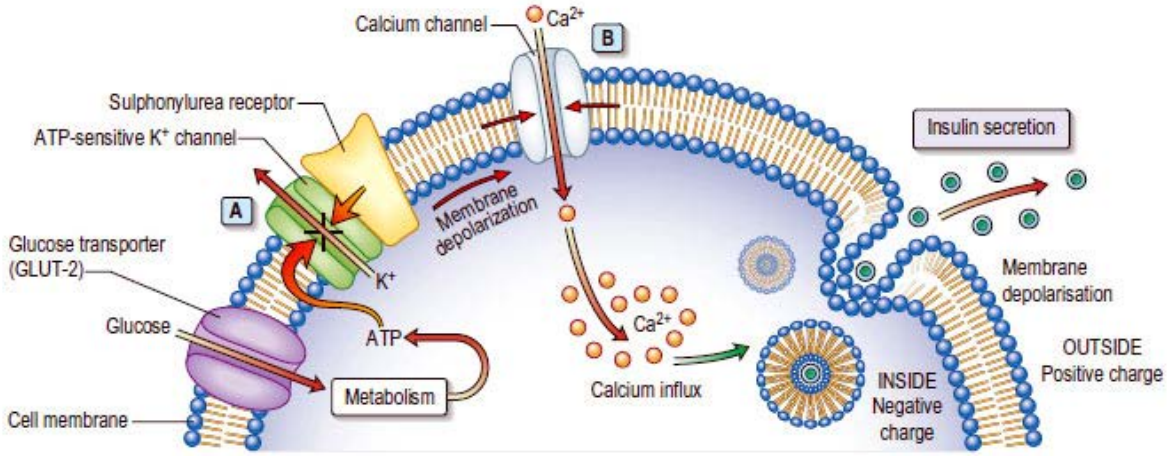

Figure 1-4 Regulation of insulin secretion (Reproduced with kind permission from medicinehack.com)

Briefly, β-cells sense glucose via the transmembrane Glucose Transporter-2 (GLUT-2), leading to ATP production via glucokinase and subsequent closure of ATP-sensitive potassium channels (Ashcroft and Rorsman, 2012). This drives calcium influx into the β-cell via voltage-gated calcium channels, which then triggers exocytosis of insulin-containing secretory granules to the cell membrane, followed by insulin release. Insulin secretion in response to rise in serum glucose after eating exhibits a biphasic response, with a rapid initial peak (first phase of insulin secretion) within 3-10 minutes, followed by a more indolent second secretory phase until normoglycaemia is restored (Zou et al., 2014). In addition to glucose, insulin release is triggered by a variety of other physiological stimuli, including gastrointestinally-derived incretins glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), parasympathetic release of acetylcholine, various amino acids

such as arginine and leucine, cholecystokinin (CCK), and drugs such as sulfonyulureas (Layden et al., 2010).

1.3.2 Insulin signaling

The metabolic effects of insulin are mediated via the insulin receptor (INSR), which is ubiquitously expressed in humans. The INSR is a member of the ligand-activated receptor and tyrosine kinase family of transmembrane signaling proteins, and is encoded by the INSR gene on Chromosome 19 (Lee and Pilch, 1994). The insulin receptor is a tetramer, consisting of two α- and two β-subunits. The α-subunits are allosteric inhibitors of β-subunit activity; once insulin binds to the former, allosteric inhibition is removed, enabling the β-subunits to autophosphorylate within the cytosol by means of intrinsic tyrosine kinase activity (Menting et al., 2014) (Figure 1-5). Insulin signal transduction is mediated via insulin receptor substrate (IRS) proteins, of which four have been identified (Rhodes and White, 2002). These IRS proteins contain a large number of tyrosine and serine phosphorylation sites. IRS activity is positively regulated by the degree of tyrosine phosphorylation, and can be negatively regulated by tyrosine phosphatases (Knobler and Elson, 2014). Most serine phosphorylation also results in negative regulation of IRS function. Tyrosine phosphorylation at residues 612 and 632 in humans is required for activation of phospho-inositide 3 kinase (PI3K).

In addition to the IRS pathway, insulin signaling also proceeds through Grb2/SHC and ras, leading to activation of the mitogen-activated protein (MAP) kinase isoforms ERK1 and ERK2 (Cheatham et al., 1994). Activation of this pathway mediates the effects of insulin on differentiation, mitogenesis, cell growth and survival.

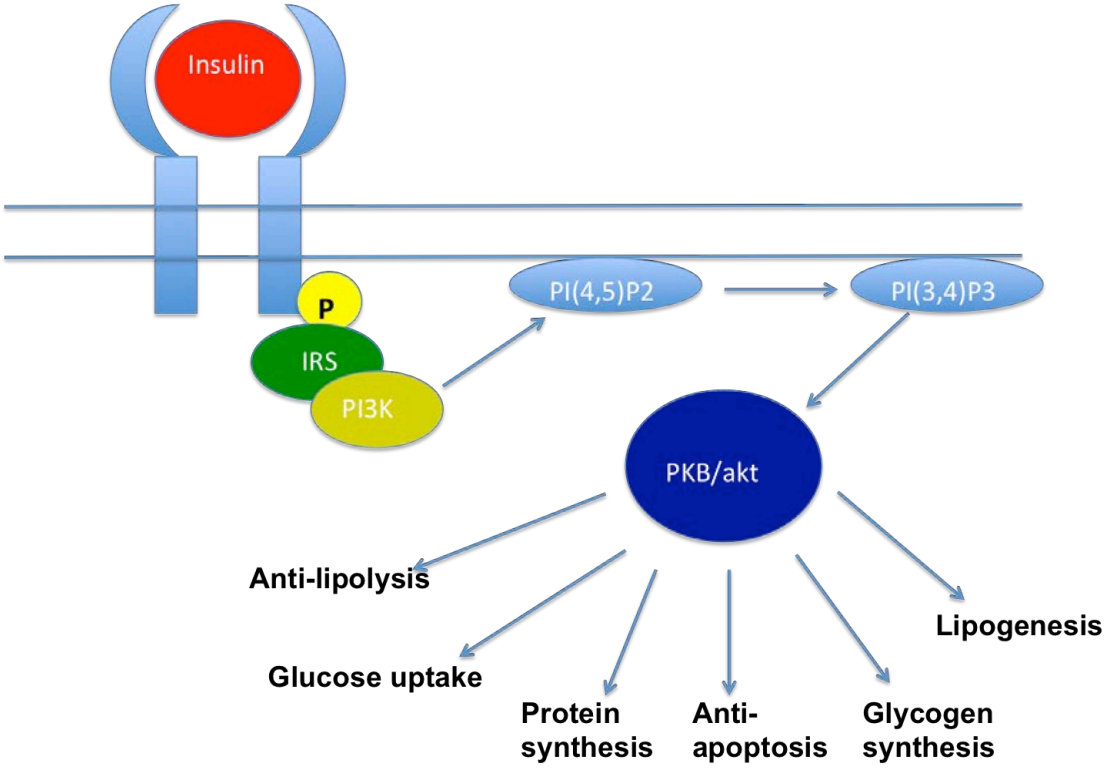

Figure 1-5 Metabolic actions of insulin signalling mediated via the INSR and PI3K pathway activation.

Once activated by IRS, PI3K mediates most of the key metabolic effects of insulin. After activation, PI3K generates phosphatidylinositol-3,4,5-triphosphate (PIP3) from phosphatidylinositol-3,4 bisphospate (PIP2). PIP3 is a secondary messenger, and is capable of activating various dependent kinases, one of the most crucial of which is protein kinase B/(PKB/akt) (Schultze et al., 2011) (Figure 1-5), of which there are three isoforms. Once phosphorylated (in the catalytic domain at Thr308 and in the hydrophobic motif at Ser473), PKB/akt is an important mediator of insulin action (Sarbassov et al., 2005), including roles in glucose uptake, transcriptional regulation, secondary messenger systems and enzymatic activation (Dummler and Hemmings, 2007). PKB/akt also phosphorylates Foxo1, inhibiting its transcriptional activity; this results in suppression of hepatic gluconeogenesis and increased cardiac myocyte survival (Battiprolu et al., 2012).

1.3.3 Metabolic actions of insulin

Insulin is released in response to a variety of stimuli, the most potent of which is plasma glucose as described above. It plays a crucial role in carbohydrate and fat metabolism, and its most important target organs are the liver, muscle and adipose tissue. As an anabolic hormone, insulin promotes fuel storage and decreases fuel mobilization. Key metabolic effects on target organs are summarized below.

1.3.3.1 Carbohydrate and glucose metabolism

The liver is an important organ of glucose generation in times of fasting. Key processes such as glycogenolysis, which is the breakdown of glycogen to release glucose, and gluconeogenesis, which involves *de novo* glucose synthesis from amino acids, glycerol, lactate and other precursors, help to maintain plasma glucose in the normal range and ensure that the body has sufficient fuel supplies in the fasted state (Rui, 2014). Insulin secretion in response to a glucose load after eating (‘fed state’) suppresses hepatic glycogenolysis and gluconeogenesis, thereby preventing development of hyperglycaemia. Glycogen storage is promoted in the liver and also muscle. Insulin also directly lowers serum and extracellular glucose levels by increasing cellular glucose uptake in adipose tissue and muscle. This process crucially depends on PKB/akt activation, which results in translocation of Glucose Transporter-4 (GLUT-4) to the transmembrane region of the cell, leading to glucose uptake in fat or muscle. GLUT-4 is localised to cytoplasmic vesicles; after insulin binds to the INSR, PKB/akt is phosphorylated, which in turn phosphorylates AS160 (Castorena et al., 2014). AS160 phosphorylation then allows vesicle translocation to the cell membrane, where GLUT-4 facilitates increased glucose uptake.

1.3.3.2 Insulin and lipid metabolism

Insulin is a crucial mediator of the balance between lipid accumulation and breakdown, or so called 'lipid flux' (Gathercole et al., 2011). In general, insulin promotes lipid accumulation by increasing lipogenesis and reducing lipid mobilization. Specifically, insulin (i) increases *de novo* synthesis of triacylglycerol (TAG), (ii) increases free fatty acid (FFA) uptake from dietary lipid and (iii) drives re-esterification of FFAs with glycerol to produce TAG, while at the same time suppressing lipolysis, which is the breakdown of TAG into FFAs and glycerol. Insulin also suppresses β-oxidation, another key process in lipid mobilization whereby FFA molecules are degraded in the mitochondria to generate acetyl-coA (Koliaki and Roden, 2013).

As discussed in section 1.3.3.1, insulin stimulates glycogen production in the liver. However, at high levels of glycogen accumulation, further glycogenesis is suppressed; at this point, additional hepatic glucose is converted into fatty acids, which are exported from the liver as lipoproteins.

1.3.3.3 Other insulin-mediated effects

In its role as an anabolic hormone, insulin stimulates amino acid synthesis and uptake into cells in the fed state. In the fasted state when insulin levels are low, the balance is tipped in favour of protein degradation (Perry et al., 2014). Insulin also exerts crucial roles in cell survival and differentiation through the MAP kinase pathway, promoting growth and gene expression.

1.3.4 Molecular features of insulin resistance

Key roles for IRS-1 and IRS-2 in growth and nutrient homeostasis have been highlighted by tissue-specific gene knockout studies in mice (Biddinger and Kahn, 2006). Simultaneous knockout of IRS-1 and IRS-2 is embryonically lethal; systemic IRS-1 knockout results in growth retardation and peripheral insulin resistance with compensatory hyperinsulinaemia, while IRS-2-null mice develop severe insulin resistance followed by pancreatic β-cell failure and hyperglycaemia (Withers et al., 1998). Tyrosine phosphorylation of the INSR and IRS-1 is impaired or absent in muscle in obesity and T2DM (Cusi et al., 2000).

Loss of IRS-1 and IRS-2 results in impaired PI3K and PKB/akt activation, leading to insulin resistance and hyperglycaemia. However MAP kinase pathway activation may be normal or even upregulated in this setting, promoting mitogenesis, overgrowth and obesity. These findings suggest differential regulation of PI3K and MAP kinase signaling pathways in the setting of insulin resistance, particularly in muscle and adipose tissue (Cusi et al., 2000). Inhibition of PI3K cascades in the liver in humans leads to hyperinsulinaemia, glucose intolerance and dyslipidaemia (Miyake et al., 2002).

Impaired PKB/akt phosphorylation also upregulates increased Foxo1 transcriptional activity, leading to increased hepatic glucose output and hyperglycaemia. In animal models, we know that hepatic knockout of Foxo1 may reverse established hyperglycaemia and heart failure in mice (Battiprolu et al., 2012). Once insulin resistance is established at a cellular level, compensatory hyperinsulinaemia may drive a sequence of molecular events with further adverse effects on insulin sensitivity. Amongst other effects, hyperinsulinaemia may suppress IRS-2 gene transcription in the liver, impair GLUT-4 cellular trafficking and paradoxically increase Foxo1 transcription (Gonzalez et al., 2011, Qi et al., 2013).

1.3.5 Monogenic disorders of insulin resistance

Insulin resistance is generally clinically expressed only in the context of obesity. In a small proportion of patients, however, severe insulin resistance (SIR) develops independent of adiposity due to monogenic gene defects impacting on either insulin signaling or adipocyte biology (Semple et al., 2011). Clinical suspicion of monogenic disorders of insulin resistance or T2DM should always be raised in insulin resistant patients with normal body mass index (BMI) or with a strong family history of insulin resistance or diabetes in first-degree relatives. SIR should be particularly considered in patients with BMI <30kg/m^2 in the setting of a fasting serum insulin >150pmol/l, and/or a peak insulin >1500pmol/l during OGTT. Symptomatic fasting or postprandial hypoglycaemia may precede onset of hyperglycaemia in severe monogenic insulin resistance by many years, with onset of the latter only after β-cell decompensation is complete. In insulin-treated patients with a BMI <30kg/m^2, a daily exogenous insulin requirement of >3 units/kg is also suggestive of SIR.

Other clinical indicators of monogenic SIR include the presence of acanthosis nigricans (dark, velvet-like thickening of the skin in the axilla, groin and skin folds characterised histologically by hyperkeratosis, papillomatosis and occasionally hyperpigmentation, Figure 1-6), or in young women by the presence of severe hyperandrogenism (Poretsky et al., 1999). Concomitant ovulatory dysfunction, androgen excess and severe PCO on ultrasound frequently results in a misdiagnosis of common PCOS in such patients. Androgen excess and PCO in patients with SIR has traditionally been interpreted as the result of synergy between insulin and gonadotrophin action at the level of the ovary, although we also know that insulin can exert direct effects on ovarian steroidogenesis (Nestler and Jakubowicz, 1996, Musso et al., 2005). However other pathways of androgen generation in these patients remain to be elucidated.

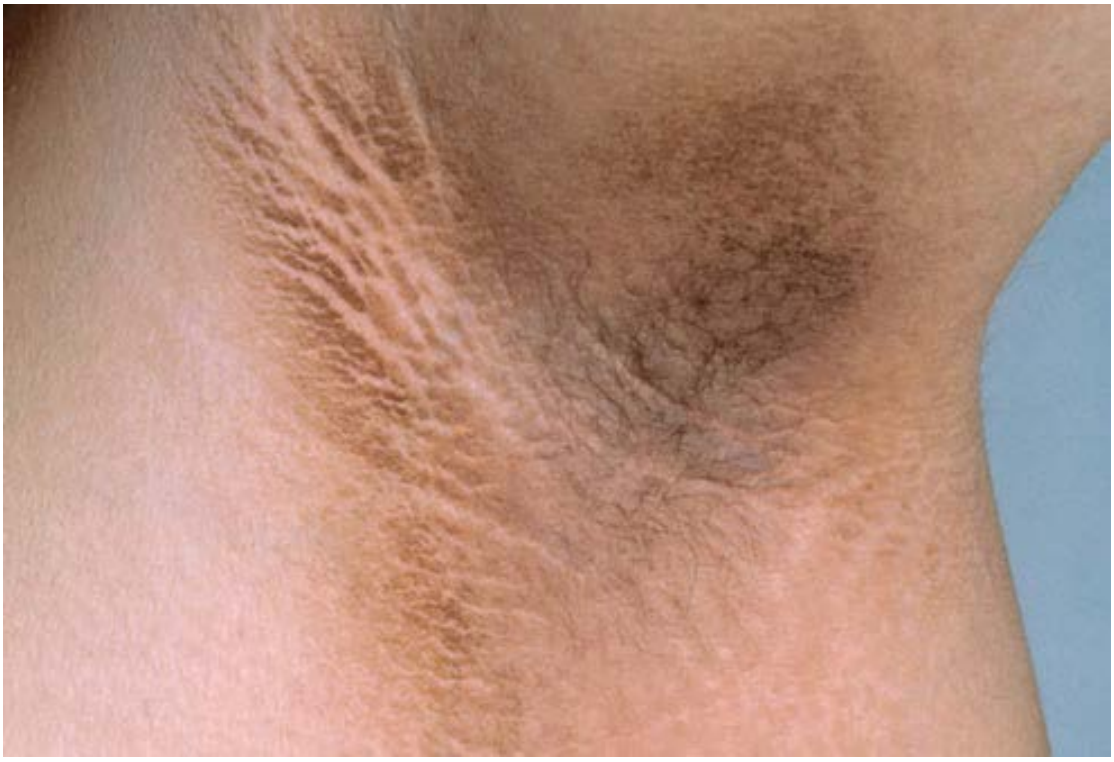

Figure 1-6 Axillary acanthosis nigricans in a patient with severe insulin resistance (SIR). (Reproduced with kind permission from WebMD UK. Copyright © 2009 Boots UK Limited and WebMD UK Limited All rights reserved).

The generic term HAIR-AN (hyperandrogenism-insulin resistance-acanthosis nigricans) is often used to describe the typical features of female patients with SIR; 'type A insulin resistance syndrome' also encompasses lean women with SIR, acanthosis, anovulation and severe androgen excess (Semple et al., 2010). A newer classification system for syndromes of SIR is proposed in Table 1-2. Monogenic insulin resistance may be categorized into disorders with primary defects in insulin signaling and abnormalities of adipose tissue development (Semple et al., 2011). Disorders of insulin signaling may be further subdivided into (i) those with defects at the level of the INSR (eg loss-of-function mutations of the INSR, so called 'insulin receptoropathies) and (ii) disorders of postreceptor insulin signal

transduction. Disorders of adipose tissue development include generalized and partial lipodystrophy, and rare genetic causes of severe obesity with hyperphagia. The section below will focus on insulin receptoropathies and lipodystrophy.

Table 1-2 Classification of syndromes of severe IR

Syndromes	**Discriminant features**
1. Primary defects in insulin signaling	
(i) Generalised (eg. INSR mutations, anti-INSR antibodies)	Severe hyperinsulinaemia; normal lipid profile; absence of hepatic steatosis; normal or increased leptin, adiponectin, SHBG
(ii) Partial (*AKT2, AS160, TBC1D4*)	Variable according to precise signaling defect
2. Secondary to adipose tissue abnormalities	
(i) Severe obesity (eg *POMC, LEP, LEPR, MC4R*)	Severe hyperphagia, early onset obesity
(ii) Lipodystrophy Generalised (eg *AGPAT2, BSCL2, PTRF, CAV1*) Partial (eg LMNA, PPARG)	Congenital absence of adipose tissue, or regional abnormalities of fat distribution Severe dyslipidaemia and hepatic steatosis Low adiponectin, leptin and SHBG levels

1.3.5.1 Insulin receptoropathies

Over 100 allelic variant defects in the INSR have been reported to date (Parker and Semple, 2013). INSR defects give rise to a heterogeneous clinical phenotype, with a spectrum of clinical and biochemical severity depending on the degree of INSR loss-of-function. Broadly speaking, insulin receptoropathies can be categorized as autosomal recessive or dominant. Recessive disorders are less common but more aggressive, generally presenting in the first decade clinically with linear growth retardation, soft tissue overgrowth and

hyperglycaemia (Musso et al., 2004). In the early stages of the disease, biochemical characteristics include fasting hypoglycaemia, postprandial hyperglycaemia and severe hyperinsulinaemia, with progression to frank hyperglycaemia often resistant to insulin treatment. Seminal clinical descriptions of such patients have lead to persistence of the older arbitrary divsion of these autosomal recessive conditions into Donohue syndrome (also termed 'leprechaunism') and Rabson Mendenhall syndrome (RMS) (Rabson and Mendenhall, 1956, Donohue and Uchida, 1954).

Autosomal dominant loss-of-function of the INSR usually presents in early puberty in females, with menstrual disturbance, hyperandrogenism and acanthosis nigricans. Male patients are often not diagnosed until the development of frank hyperglycaemia in the 4th or 5th decade of life; earlier diagnosis in males may be facilitated by targeted familial screening after the diagnosis of a first-degree relative. As summarized in Table 2, patients with receptor defects in insulin signaling are protected from the severe dyslipidaemia and fatty liver disease seen in patients with lipodystrophy or 'post-receptor' insulin signaling defects (Semple et al., 2009).

1.3.5.2 Lipodystrophy

Defects in adipose tissue development are considered a secondary cause of SIR. Lipodystrophy may be categorized as generalized or partial, inherited or acquired. Acquired partial lipodystrophies include HIV-associated lipodystrophy and C3 nephritic factor-associated lipodystrophy (Grinspoon, 2005, Misra et al., 2004). Lipodystrophy is relatively easily identified clinically, and should be considered in all patients with SIR with derangements in lipid profile and biochemical or radiological evidence of non-alcoholic fatty liver disease (NAFLD).

Congenital generalized lipodystrophies (CGL), such as Berardinelli-Seip congenital lipodystrophy (BSCL) are characterized by an almost complete lack of adipose tissue from birth, with early onset of insulin resistance in infancy, followed by overt hyperglycaemia in the 2nd or 3rd decade (Garg, 2011). CGL has a striking clinical phenotype and is usually apparent at birth. Partial lipodystrophies, such as familiar partial lipodystrophy, are characterized by adipose tissue atrophy in distinct depots, usually in the limbs, with variable truncal fat loss and often excess fat around the face or neck (Prieur et al., 2014). The phenotype in partial lipodystrophy may be more subtle, but should be considered in lean patients with hepatic steatosis, severe dyslipidaemia, acanthosis and, in women, severe hyperandrogenism and PCO.

1.4 Physiology of androgen secretion and androgen excess in females

1.4.1 Androgen biosynthesis

Androgen biosynthesis in females occurs predominantly in the adrenal glands and ovaries. In the adrenal glands, androgen generation in the zona reticularis of the adrenal cortex is driven by adrenocorticotrophic hormone (ACTH), while luteinizing hormone (LH) stimulates *de novo* androgen generation in ovarian thecal cells (Burger, 2002). Androgen activation from weak precursors to potent T and dihydrotesterone (DHT) in peripheral tissues is also increasingly recognized; peripheral androgen generation in adipose tissue, liver, skin and other 'intracrine' organs is now believed to play an important role in androgen metabolism in health and disease (Fassnacht et al., 2003, Quinkler et al., 2004). Adrenal and

ovarian androgen synthesis will be discussed below. Adipose androgen metabolism is discussed in section 1.7.

1.4.1.1 Adrenal androgen synthesis and metabolism

The adrenal glands are a crucial organ of androgen biosynthesis in humans. The adrenals are located bilaterally in the retroperitoneum, sitting just above the kidney on both sides; each gland weighs approximately 4 grams (McNicol, 2008). The adrenal gland is composed of an outer cortex and inner medulla; the latter is responsible for synthesizing and secreting catecholamines, such as adrenaline, noradrenaline and dopamine. The adrenal cortex is composed of three layers: the outermost zona glomerulosa is the site of mineralocorticoid synthesis; the middle layer, the zona fasciculata, synthesizes glucocorticoids such as cortisol; the innermost zona reticularis is responsible adrenal androgen synthesis and release (Idkowiak et al., 2011) (Figure 1-7).

The adrenal androgen precursors dehydroepiandrosterone (DHEA) and its sulfated ester DHEAS are secreted by the zona reticularis. DHEA is sulfated to inactive DHEAS by the action of cytosolic sulphotransferase (SULT2A1*)*; conversely DHEAS can be hydrolysed to DHEA by microsomal steroid sulphatase (STS) (Oostdijk et al., 2015). Approximately 75% of circulating DHEA is derived from the adrenal gland (either directly synthesised or derived from circulating DHEAS); the remainder is produced in the gonads (Longcope, 1986). DHEA may be converted to androstenedione (A) by 3β-HSD2 (*HSD3B2*) in both the adrenal and ovary, and subsequently to more potent androgens such as T and DHT in peripheral target tissues (see below).

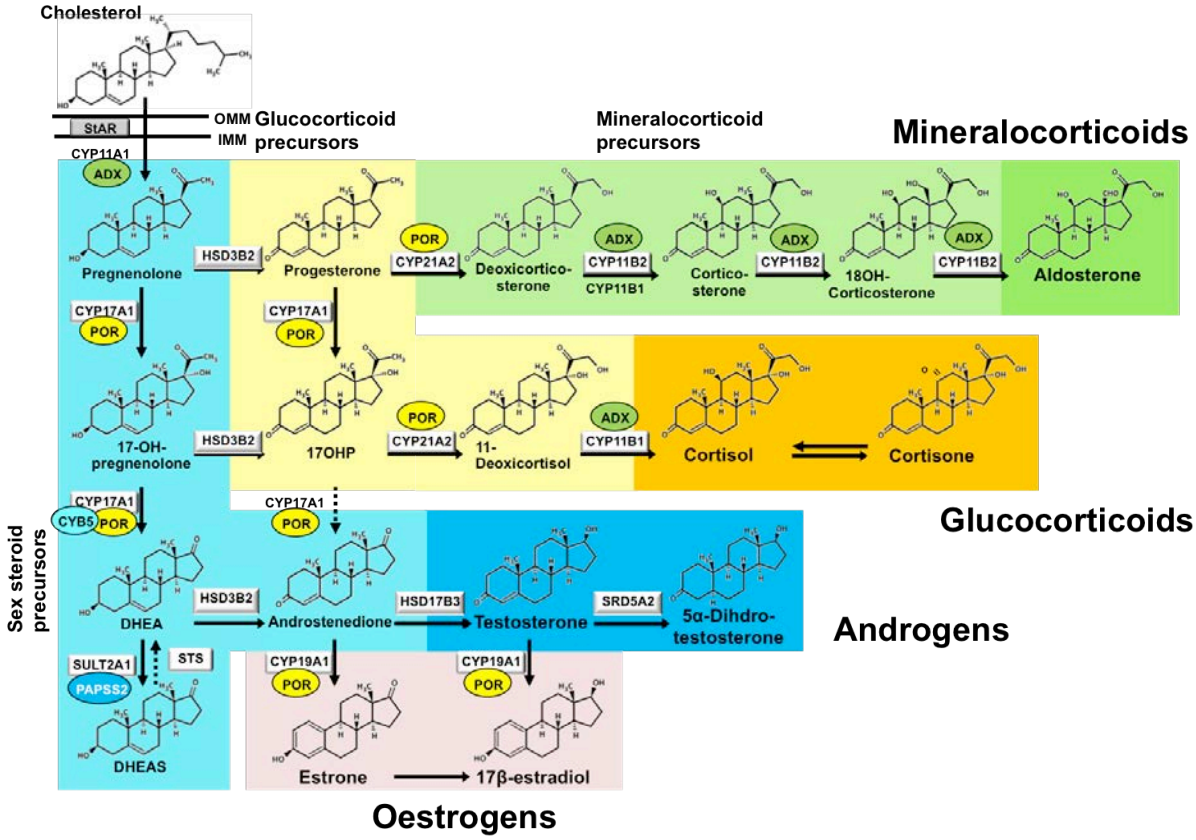

Figure 1-7 Adrenal steroidogenesis from cholesterol precursor. Mineralocorticoids are synthesized in the outermost zona glomerulosa (light green). Glucocorticoid synthesis occurs in the zona fasciculata (yellow), while the innermost zona reticularis (pale blue) synthesises and secretes the adrenal androgens DHEA and DHEAS. DHEA is metabolized to active androgens in peripheral target tissues. Androstenedione and testosterone may be also be peripherally aromatised to oestrogens by CYP19A1 (placental aromatase).

Circulating concentrations of DHEA and DHEAS exhibit a characteristic age-associated pattern (Arlt, 2004) (Figure 1-8). High levels in the perinatal period reflect fetal adrenal androgen production, but levels decline rapidly after involution of the fetal zone of the adrenal cortex. Levels of DHEAS rise again between 6th and 10th year of life, reflecting a process of maturation of the zona reticularis known as adrenarche (Sklar et al., 1980), before peaking in the third decade. There is a steady decline in DHEAS thereafter, dropping to 10-

20% of maximum levels by the eighth decade (Orentreich et al., 1992). This age-related drop in adrenal androgens has been termed the 'adrenopause', attributed to involution of the zona reticularis, and occurs in both men and women, independent of the menopause.

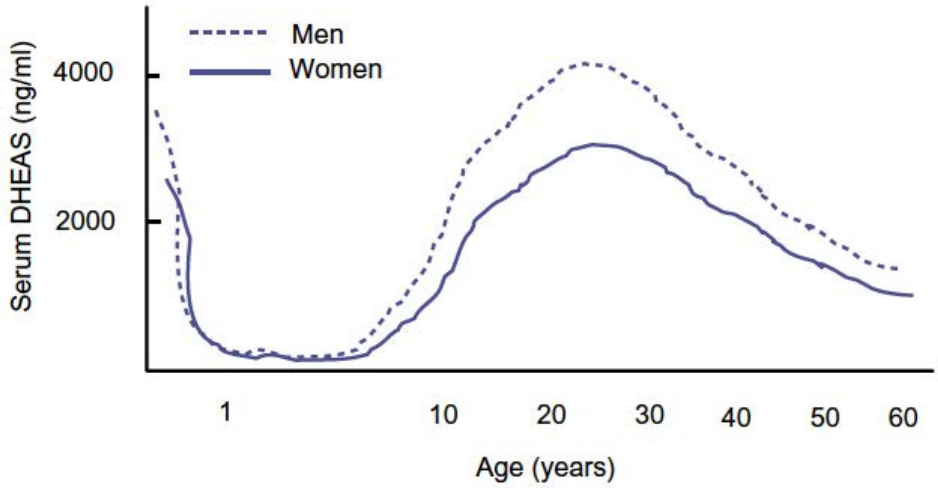

Figure 1-8 Serum DHEAS levels in men and women throughout life (Arlt, 2004). Reproduced with permission from Elsevier.

1.4.1.2 Ovarian androgen synthesis and metabolism

The ovary is another crucial site of androgen generation in women. Ovarian androgen generation takes place in the outer theca cells of the follicular unit, and is regulated by pituitary LH secretion. Each basic follicular unit contains a single oocyte and granulosa cells, which are separated from the outer theca cells by a basement membrane or basal lamina (Magoffin, 2005). Theca cells form a layer of cells adjacent to the basal lamina known as the theca interna; this area is highly vascularized and steroidogenic (Erickson et al., 1985) (Figure 1-9***)***. Primordial follicles do not contain theca cells, and the latter only begin to differentiate in the theca interna after a second layer of cuboidal granulosa cells develops in the second stage

of follicular development. Upon reaching steroidogenic potential, theca cells are responsive to LH stimulation.

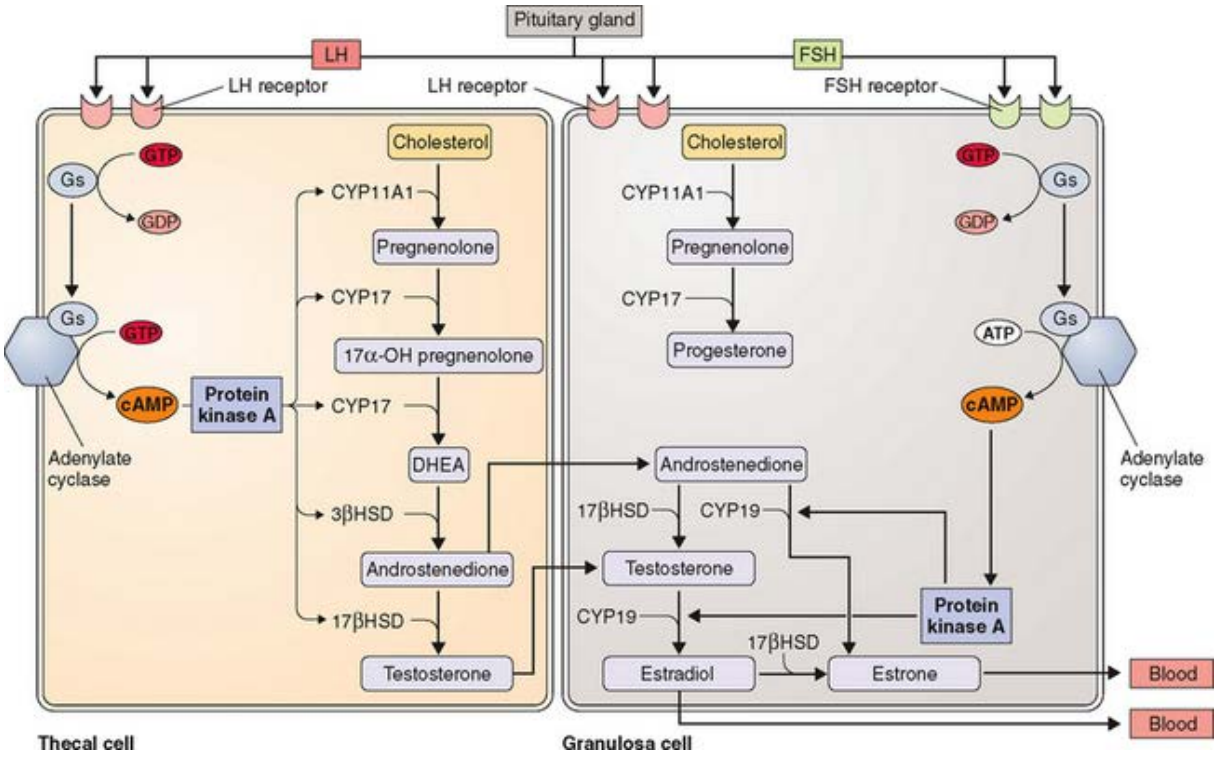

Figure 1-9 Theca and granulosa cell action under the influence of FSH and LH across follicular development in the human ovary (Reproduced with kind permission from ClinicalGate.com).

De novo thecal androgen generation is dependent upon expression of a number of key steroidogenic enzymes, a process driven by differentiation of the theca interna during folliculogenesis (Logan et al., 2002). LH receptor expression facilitates expression of *CYP11A1*, *HSD3B2* and *CYP17*. *CYP11A1* (cholesterol side-chain cleavage enzyme) is localized to the mitochondria, while *CYP17* and *HSD3B2* are found in the endoplasmic reticulum. LH drives thecal androgen generation through stimulation of the cAMP-signaling pathway, leading to expression and upregulation of *CYP17* and *HSD3B2*. LH also upregulates

steroidogenic acute regulatory protein (StAR), a key protein involved in the transport of cholesterol to the inner leaflet of the mitochondria, where it acts as substrate for *CYP11A1* (Nelson et al., 2001). The stimulatory action of LH on ovarian steroidogenesis are modulated by a long list of growth and differentiation factors which may be derived from the ovary itself or from the peripheral circulation (Magoffin, 2002). Factors acting synergistically to augment LH activity include insulin, insulin-like growth factor-1 (IGF-1), inhibin and GDF-9; factors that inhibit LH action include activin and TGF-α. The role of insulin in this process will be discussed in more detail in section 1.5.

The principal androgen precursor product of thecal steroidogenesis is A. Androgens are important modulators of follicular function, exerting their effects via the androgen receptor (AR). The AR is expressed throughout the mature ovarian follicle, including oocyte, theca and granulosa cells (Lebbe and Woodruff, 2013). Once the follicular unit has transitioned from primordial to primary follicle, AR expression allows testosterone to stimulate further follicular maturation to the secondary follicle stage; this has been reported both in primate models and in ovaries from testosterone-treated transsexual women (Weil et al., 1998, Chadha et al., 1994). Whilst androgen action is crucial for follicular development, conditions of androgen excess such as PCOS are characterized by the accumulation of small antral follicles in the ovarian cortex. The obvious deduction from these findings is that supraphysiological androgen exposure may be detrimental in the early stages of follicular development, leading to disruption of dominant follicle selection and subsequent anovulatory fertility (Franks and Hardy, 2010). Theca cell hyperplasia is a common histological finding in ovarian tissue from women with PCOS.

The theca provides crucial androgen substrate for aromatization to oestrogens in the granulosa cells; human theca cells do not express *CYP19A1*. In order for aromatization to

proceed, A must therefore diffuse from the theca across the basal lamina to the granulosa cells, which express *CYP19A1* from the small antral stage onwards. FSH, secreted from gonadotroph cells in the anterior pituitary, significantly upregulates granulosa aromatase activity. Interestingly, this induction of granulosa aromatase activity by FSH is itself positively regulated by androgens via the AR (Hillier and De Zwart, 1981). Therefore, in addition to providing substrate for aromatization, thecal androgens themselves induce granulosa aromatase activity, allowing granulosa cells in the selected dominant follicle to synthesise large quantities of oestrogen. This process of cross-talk between theca and granulosa cells, cumulating in oestrogen synthesis by the follicular unit, and initially proposed by Bjersing in 1967, is termed the two-cell, two gonadotrophin theory (Bjersing and Carstensen, 1967); thecal androgen generation under LH stimulation provides substrate for granulosa aromatase activity which is stimulated by FSH.

1.4.2 Peripheral (pre-receptor) androgen metabolism

A large body of evidence supports activation of weaker androgen precursors in peripheral target tissues to more potent metabolites such as T and DHT. Although *de novo* androgen generation from cholesterol in the peripheral tissues is unlikely, secretion of large quantities of DHEA and DHEAS from the adrenal glands provides substrate for a complex process of peripheral androgen generation. The term ‘intracrinology’ has been coined in the last two decades to describe this process of local hormone synthesis in selected target tissues such as skin, liver, fat, muscle, cerebral cortex and breast (Labrie et al., 1997a); locally-generated hormones may then exert their effects on the tissue of origin without being released systemically or into the extracellular tissues. After menopause and consequent cessation of ovarian oestradiol secretion, the vast majority of sex steroids are synthesized locally in

peripheral intracrine tissues. However, declining serum levels of DHEA and DHEAS with advancing age ('adrenopause') may lead to a global reduction in substrate available to peripheral tissues; decreased local androgen synthesis in target organs may play a role in age-related diseases such as sarcopenia (Labrie et al., 2003).

Tissue-specific androgen generation is dependent upon local expression and activity of key steroidogenic enzymes. Peripheral metabolism of DHEA to more potent androgens requires 3β-HSD, 17β-HSD and 5αR enzymatic activity (Figure 1-10***)***. Additionally, A and T may be further aromatized to oestrone and oestradiol, respectively, in the presence of placental aromatase (*CYP19A1*). DHT, however, is not susceptible to aromatization. 3β-HSD is responsible for conversion of DHEA to A. Type 2 3β-HSD (*HSD3B2)* is largely expressed in the adrenals and ovaries (Luu-The et al., 1991); the most important peripheral 3β-HSD isoform appears to be type 1 (*HSD3B1*), which is expressed in skin, adipose tissue, liver, brain and breast (Milewich et al., 1993). There are multiple isoforms of human 17β-HSD; types 3 and 5 are the most important for androgen activation, converting A to T (Dufort et al., 1999). Type 3 17β-HSD is expressed predominantly in the Leydig cells of the testes, contributing to approximately 50% of total androgen load in men. It shares significant homology with type 5 17β-HSD, which catalyses the conversion of A to T in peripheral tissues. Type 5 17β-HSD is expressed in a variety of tissues, including adrenal, ovary, adipose and breast (Quinkler et al., 2004). It belongs to the aldoketoreductase family of enzymes, and is also called aldoketoreductase type 1C3 (AKR1C3). The role of this enzyme in adipose androgen metabolism will be discussed in detail in Section 1.7.

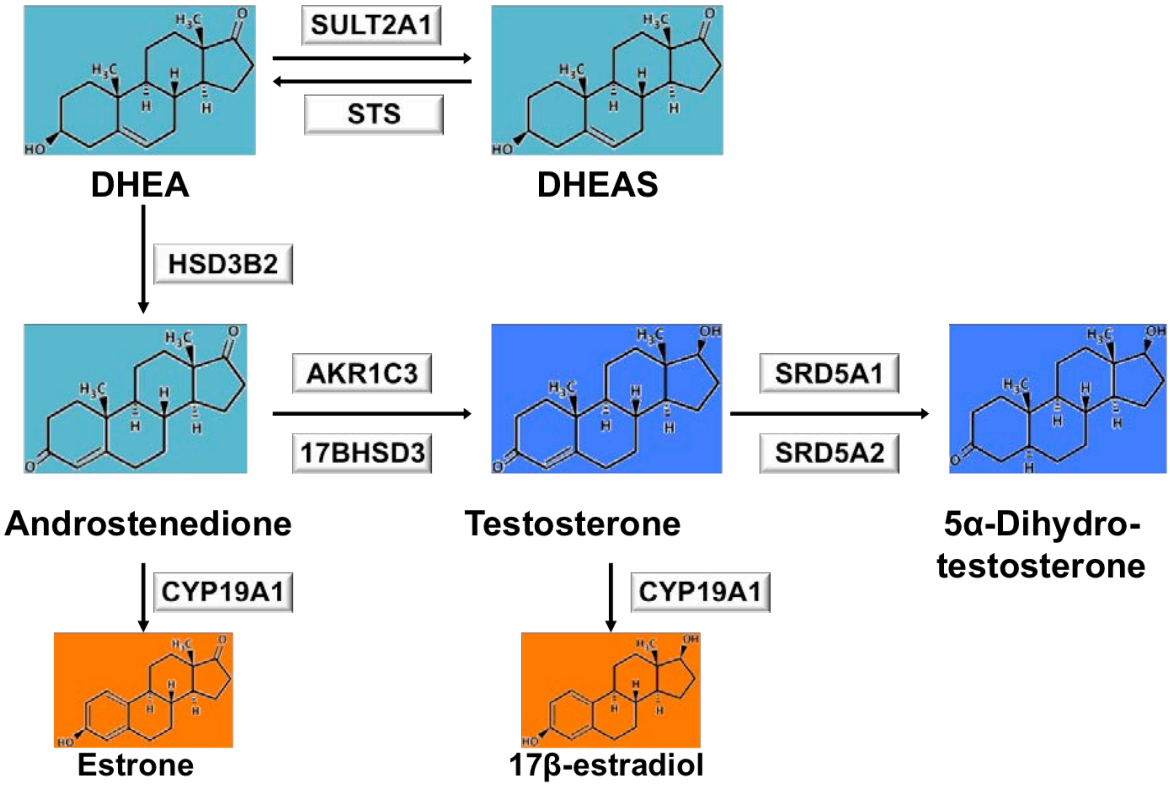

Figure 1-10 Peripheral pre-receptor androgen metabolism. 3β-HSD type 1(HSD3B1) catalyses the peripheral conversion of DHEA to androstenedione (A), which in turn is converted by 17β-HSD5 (also called AKR1C3) to testosterone (T). T may bind to the androgen receptor directly or undergo conversion to potent dihydrotestosterone (DHT) by the action of 5α-reductase type 1 (SRD5A1) or type 2 (SRD5A2). A and T may be aromatized to oestrone (E1) and oestradiol (E2) respectively.

5αR catalyses the conversion of T to DHT, the most potent androgen, which binds to the AR with significantly greater affinity than T. The enzyme 5αR has three isoforms, with isoforms 1 and 2 sharing 47% homology. *SRD5A1* is expressed predominantly in skin (in particular genital) and adipose tissue, and *SRD5A2* in reproductive tissues such as the seminal vesicles, prostate and epididymis in men, and ovaries in women; both isoforms are expressed in the liver (Tomlinson et al., 2008b). 5αR2 is likely to be the most important contributor to androgen activation in humans, but the role of 5αR1 in diseases such as PCOS and obesity

remains to be elucidated. Increased expression of 5αR1 in the skin causes hirsutism (Dijkstra et al., 1987). The role of 5αR3 is poorly understood and it is unlikely to be a key player in systemic androgen or glucocorticoid metabolism.

In vivo evidence for peripheral androgen formation comes from studies examining dexamethasone-induced suppression of adrenal androgens in PCOS women compared to controls; androgens remain higher in the PCOS cohort despite administration of dexamethasone (Lachelin et al., 1982). Mirroring this, suppression of ovarian androgen production using long-acting gonadotrophin-releasing hormone (GnRH) agonists results in significant suppression of androgen levels in both groups; however post-treatment androgen levels remain persistently higher in PCOS patients (Rittmaster and Thompson, 1990). Peripheral androgen generation may make an important contribution to androgen excess in PCOS. Stewart *et al* identified evidence of systemically upregulated urinary excretion of 5α-reduced androgen and glucocorticoid metabolites in women with PCOS (Stewart et al., 1990). This provides indirect evidence of increased peripheral 5α-reductase (5αR) activity in this disorder. Another key study showed that urinary excretion of the 5α-reduced metabolite androsterone (An) was significantly increased in PCOS compared to controls after administration of an oral androgen load with DHEA, mirrored in the serum by increased levels of DHT (Fassnacht et al., 2003). Interestingly, Vassiliadi and colleagues found that peripheral 5α-reductase activity, as measured indirectly using the same urinary ratios described in the Stewart paper, correlated closely with serum insulin levels and markers of insulin resistance (Vassiliadi et al., 2009). The androgen-activating and glucocorticoid-inactivating roles of 5α-reductase are shown in Figure 1-11.

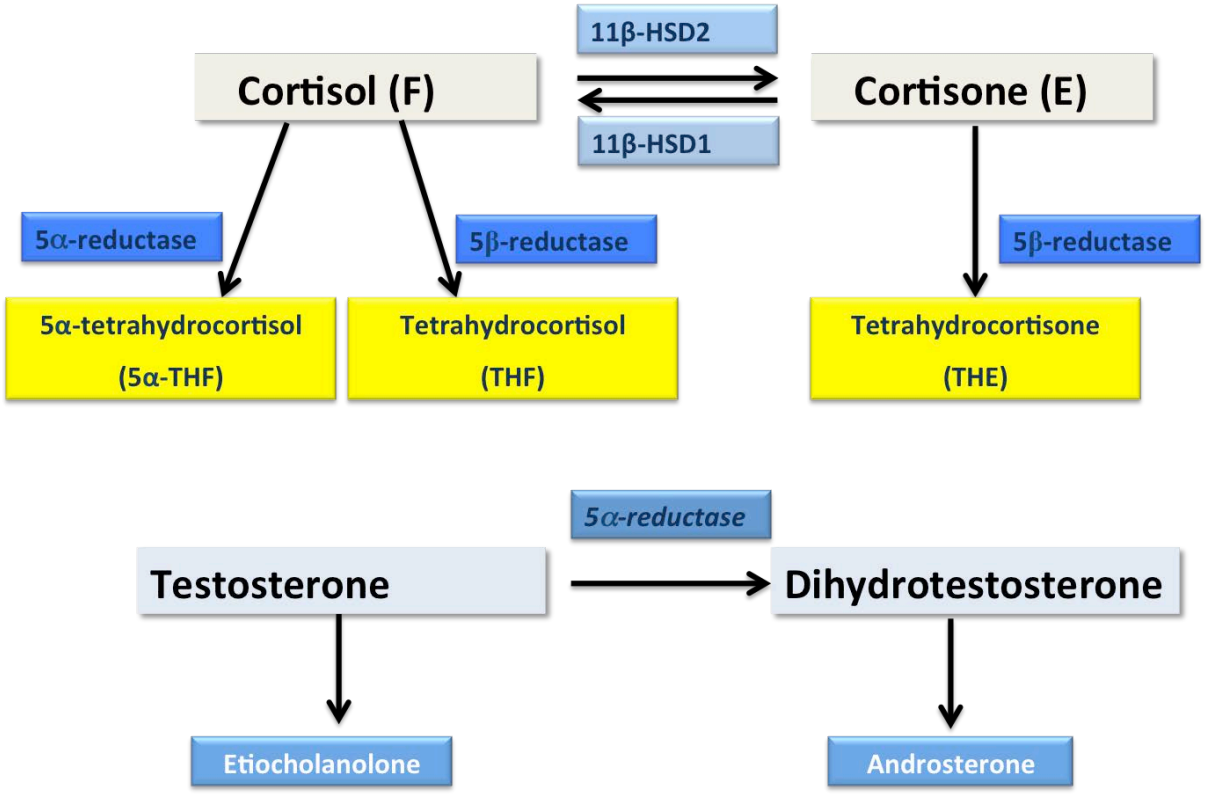

Figure 1-11 Androgen activation and glucocorticoid inactivation by 5α-reductase. Net systemic 5α-reductase activity is calculated in urinary steroid excretion profiles by the ratio of androsterone to etiocholanolone (An/Et) and 5α-THF/THF. 11β-HSD1 activity is estimated by the ratio of (5α-THF+THF)/THE.

1.4.3 The role of androgens in female health and development

1.4.3.1 Androgen receptor (AR)

The biological effects of androgens are mediated in both sexes via the androgen receptor (AR), a member of the nuclear receptor superfamily (subfamily 3) (Montgomery et al., 2001). Other members of this family include the mineralocorticoid, glucocorticoid, oestrogen and progesterone receptors. The AR gene is located on the X-chromosome (Xq11-12). The AR (919 aa) consists of four domains: 1) a large N-terminal domain (NTD) with an activation function 1 (AF1), 2) a DNA-binding domain (DBD), 3) the hinge region (HR)

which contains a nuclear localization signal (NLS), and 4) a ligand-binding domain (LBD), containing the activation function 2 (AF2) (Figure 1-12).

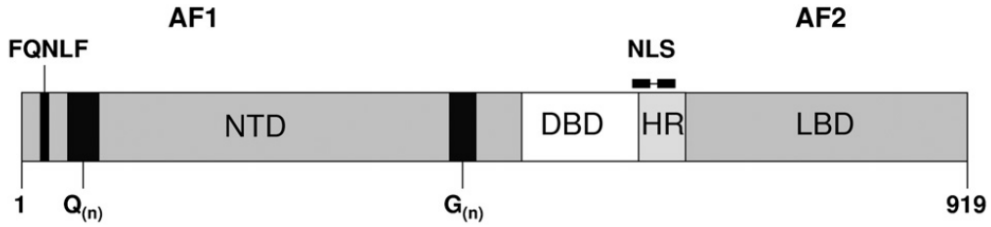

Figure 1-12 Protein structure and domains of the androgen receptor (AR) (Werner et al., 2010). NTD: N-terminal domain; DBD, DNA-binding domain; HR, hinge region; LBD, ligand-binding domain; AF1/2, activation function 1 and 2; NLS, nuclear localization signal; Q(n), polymorphic glutamine repeat; G(n), polymorphic glycine repeat; FQNLF, peptide interacting with AF2. Reproduced with permission from Elsevier.

In the absence of ligand, the AR is largely confined to the cytoplasm. After binding of T or DHT, the AR undergoes conformational change, leading to dissociation of heat shock proteins (HSPs), which enables nuclear targeting of AR and subsequent nuclear dimerization (Bennett et al., 2010). Androgen response elements (AREs) promote recruitment of co-activators, which facilitate subsequent gene transcription. Non-ligand bound AR is returned to the cytoplasm where it is either recycled in preparation for further ligand binding, or targeted for ubiquitin-mediated proteasomal degradation.

1.4.3.2 Androgens and fetal sexual differentiation

Androgens exert multi-system biological actions in humans; these effects may be gender-specific. In embryogenesis, androgens play a key role in early development of external genitalia (sex differentiation). This is mechanistically distinct from the earlier phase of sexual determination, which is controlled by transcription factors and non-steroid regulators. Sexual

dimorphism occurs at about 6 weeks after conception, when androgens drive differentiation of the bipotential gonadal anlage into testes. Low levels of circulating androgens in the female fetus allow the development of the genital tubercle and urogenital swellings into clitoris and labia majora, respectively (Hiort, 2013). In complete androgen insensitivity syndrome (CAIS), affected 46,XY individuals have a deleterious loss-of-function mutation of the AR, which results in external genitalia with *in utero* resistance to the virilising effects of androgens (Werner et al., 2010). Such patients have phenotypically female external genitalia with clitoris and labia majora. Mirroring this, 46,XX individuals with disorders of adrenal steroidogenesis, such as congenital adrenal hyperplasia (CAH), may be exposed to excess adrenal androgens during crucial stages of embryogenesis, leading to virilisation of the external genitalia. Severe cases may result in complete masculinization, with clitoromegaly, scrotum formation from the urogenital swellings and even frank phallus development (Kamrath et al., 2013).

1.4.3.3 Androgens, adrenarche, pubarche and gonadarche

As discussed in section 1.4.1.1, high circulating DHEAS levels in the early neonatal period decline rapidly in the first three months of life. Physiological increases in serum DHEAS levels occur in both sexes around 6-8 years of age, although recently more sensitive detection methods such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-tandem mass spectrometry suggest that secretion of adrenal androgens may begin as early as 3 years of age (Remer et al., 2005). This process is termed adrenarche, and, although its regulation is poorly understood, is likely to reflect maturation of the zona reticularis. DHEA secreted by the adrenal is then activated to more potent androgens in the adrenals and peripheral tissues, which leads to the development of pubic (‘pubarche’)

and axillary hair. This occurs physiologically from the age of 8 years onwards (Idkowiak et al., 2011). Adrenarche is also associated with development of a distinctive male body odour in both sexes, as well as greasy hair and skin (Voutilainen et al., 1983). Although definition consensus remains elusive, premature adrenarche (PA) is best defined by the presence of elevated adrenal androgen levels above age- and sex-matched reference interevals, with concurrent clinical evidence of increased androgen action (such as pubarche), below the age of 8 years in girls and 9 years in boys (Utriainen et al., 2007). Premature adrenarche is associated with a constellation of subsequent metabolic and reproductive abnormalities, which will be discussed further in section 1.5.

It is important to note that adrenarche appears to be a process largely independent from gonadarche, the term used to describe maturation of the gonads and onset of sex steroid production. This notwithstanding, adrenal androgens may indirectly modulate GnRH pulsatility, thereby kick-starting gonadarche. Gonadal androgen secretion manifests clinically with testicular and penile enlargement in boys, and breast development (also termed ‘thelarche’) and menarche in girls. Onset of gonadarche is reliant upon maturation of the GnRH ‘pulse generator’; increasing GnRH amplitude stimulates LH pulsatility, which drives gonadal androgen secretion (Neely and Crossen, 2014). GnRH pulse amplitude and frequency increases to every 1-2 hours, particularly at night, in early puberty (Apter et al., 1993). Initiation of GnRH release from the hypothalamus is a complex process mediated by neuropeptides such as kisspeptin, leptin and neurokinin. When this process begins prematurely, manifesting clinically as thelarche and/or menarche in girls, and testicular and/or penile enlargement in boys, it is referred to as true central precocious puberty (Fuqua, 2013). However, like premature adrenarche, there is great controversy over the issue of timing of normal pubertal development. This is largely a reflection of an increased number of children

entering puberty at younger ages, and therefore distinguishing pathological and physiological onset becomes much more difficult. A 1997 study found that the average age of thelarche in white and African-American girls was 10 years and 8.9 years, respectively (Herman-Giddens et al., 1997), but this remains controversial. Most commentators agree that precocious puberty implies the development of secondary sexual characteristics (testicular enlargement, breast development or menarche) before the age of 9 years in boys or 8 years in girls.

True central precocious puberty (CPP), often called GnRH-dependent precocious puberty, is the clinical and biochemical consequence of premature upregulation of the hypothalamic-pituitary-gonadal axis. Idiopathic CPP represents over 90% of cases and is more common in girls than boys. A variety of other subtypes of CPP exist, triggered by acquired CNS insults such as trauma, infection and neoplastic disease; it is also described in inherited or developmental CNS diseases such as Tuberous Sclerosis or Neurofibromatosis Type 1 (Fuqua, 2013). GnRH-independent precocious puberty (also termed precocious pseudopuberty) is much less common, observed in rare gonadal or adrenal androgen-secreting tumours, CAH and McCune-Albright syndrome. Differentiating CPP from PA may be difficult on clinical grounds alone, but generally girls with pubic and/or axillary hair without evidence of thelarche, or boys with pubic/axillary hair without testicular enlargement, are likely to have PA. Diagnosis may be further complicated by the existence in girls of the phenomenon of premature thelarche, in which modest early breast development is noted in the absence of menstruation and other pubertal findings. Clinical correlation with biochemical findings of pubertal range testosterone levels in boys and 17β-oestradiol in girls, as well as gonadotrophin response to GnRH stimulation, is usually sufficient to make the diagnosis.

1.4.3.4 Multi-system effects of androgens in females

Although their actions are classically associated with male sexual development, growth and general well-being, androgens also exert multi-system effects in females, and states of androgen excess or deficiency in women are associated with significant health consequences. The major physiological role played by androgens in females are highlighted by data showing that total androgen production in healthy women, as measured by 24-hour excretion of key conjugated metabolites of DHT, are up to 70% of those observed in men (Labrie et al., 1997b). As explored throughout this thesis, isolated measurement of serum androgens in females, circulating in relatively low concentrations, is a weak tool for estimation of total systemic androgen metabolism, and does not take into account the complex intracrine systems of androgen activation described in section 1.4.2. Urinary androgen metabolite excretion, perhaps in the future coupled with assessment of tissue-specific local androgen activation, represents a more comprehensive evaluation of androgen metabolism in health and disease.

An important physiological role for androgens in female bone metabolism is highlighted in both clinical and laboratory studies. Co-administration of oestrogen and testosterone implants to postmenopausal women results in a greater improvement in radiological bone mineral density at the lumbar spine than in those treated with oestrogen alone (Davis et al., 1995). Patients with complete androgen insensitivity syndrome (CAIS) have significantly reduced bone mineral density which is not ameliorated by treatment with oestrogen alone (Soule et al., 1995). Therefore the effect of androgens on bone density appear to be directly mediated via the AR and not as a consequence of aromatization to oestrogens. *In vitro*, androgens have direct effects on osteoblast differentiation and endochondral bone formation (Kapur and Reddi, 1989, Kasperk et al., 1989).

The role of androgens in cardiovascular and metabolic health in men has been extensively debated. Although little consensus exists on whether cardiovascular benefits are derived directly from testosterone replacement in obesity-induced hypogonadism, a recent meta-analysis reinforced the dogma that low serum testosterone levels at the very least represent an indirect marker of cardiovascular risk (Srinath et al., 2015). Modest testosterone supplementation may also have benefits for cardiovascular health in women. Long-term testosterone implant therapy does not appear to adversely affect the observed improvements in lipid parameters associated with postmenopausal oestrogen replacement (Davis et al., 1995). Additionally, parenteral testosterone replacement may directly induce brachial artery vasodilatation (Worboys et al., 2001).

In addition to the potential benefits described above, co-administration of androgens with hormone replacement therapy (HRT) may have benefits on quality of life and general well-being in postmenopausal women (Davis and Tran, 2001). Transdermal testosterone has been shown to improve libido and sexual function in women after oophorectomy (Shifren et al., 2000). Treatment with DHEA 50mg for four months in a study of 24 women significantly improved general well-being and sexuality compared to placebo in women with adrenal insufficiency (Arlt et al., 1999). It is important however for clinicians to recognize the delicate balance between physiological androgen replacement and the potentially deleterious metabolic effects of androgen excess in such women. This is echoed in a recent Endocrine Society clinical practice guideline, which advised against routine diagnosis of androgen deficiency in healthy women due to insufficient supporting evidence correlating androgen levels with specific signs and symptoms of disease (Wierman et al., 2014).

1.4.4 Monogenic disorders of androgen excess

A number of monogenic disorders of steroidogenesis result in clinically significant androgen excess, the consequences of which are most severe in females. Most of these genotypically and phenotypically heterogeneous conditions result in adrenal androgen excess, and fall under the umbrella term of CAH. Specific enzymatic deficiencies may indirectly lead to upregulation of adrenal androgen generation. These and other monogenic causes of androgen excess are summarized in Table 1-3**.** CAH subtypes resulting in sex steroid deficiency are not covered here. Genetic disorders of androgen excess have severe clinical consequences if untreated, and require life-long multi-disciplinary follow-up unified under an experienced adrenal endocrinologist to maximize quality of life and satisfactory long-term health outcomes (Auchus and Arlt, 2013, Han et al., 2013). The most common subtypes are discussed in more detail below.

Table 1-3 Summary of clinical and genetic features of known monogenic disorders of androgen excess.

Genetic defect and inheritance	**Impaired reaction**	**Discriminant features**
CYP21A2 (21-hydroxylase deficiency) Autosomal recessive	Progesterone→11-deoxycorticosterone 17OHP→11-deoxycortisol	**Classic**: severe GC and MC deficiency, ACTH-mediated androgen excess **Simple virisiling**: 46,XX DSD **Non-classic**: Androgen excess, compensated GC deficiency
CYP11B1 Autosomal recessive	11-deoxycortisol→cortisol	**Classic**: isolated GC deficiency, ACTH-mediated androgen excess, increased MC precursors **Non-classic**: often androgen excess only
HSD3B2 Autosomal recessive	Pregnenolone→Progesterone 17OH-preg→17OHP DHEA→androstenedione	**Classic**: GC and MC deficiency. Androgen excess with downstream conversion of accumulating DHEA to active androgens. **Non-classic:** Compensated GC/MC deficiency; androgen excess
*CYP19A1 (*non CAH; aromatase deficiency Autosomal recessive	Testosterone→E2 Androstenedione→E1 DHEA→E3	Prenatal virilisation due to impaired aromatization: 46,XX DSD
HSD11B1 (cortisone reductase deficiency) Autosomal recessive	Cortisone→cortisol	ACTH-mediated postnatal androgen excess, late presentation with PCOS or premature adrenarche
POR (P450 oxidoreductase deficiency) Autosomal recessive	All microsomal cytochrome P450 enzymes - impaired CYP17A1, CYP21A2, CYP19A1, CYP51A2 activity	GC deficiency Prenatal androgen excess Postnatal sex steroid deficiency Skeletal malformations
PAPSS2 (apparent DHEA sulfotransferase deficiency) Autosomal recessive	Impaired SULT2A1 activity: DHEA→DHEAS	Androgen excess due to impaired sulfation Bone malformations
H6PD (apparent cortisone reductase deficiency) Autosomal recessive	Impaired HSD11B1 activity: Cortisone→cortisol	Variable androgen excess, often presenting with premature adrenarche or PCOS phenotype

1.4.4.1 Principles of clinical management of adult patients with CAH

21-hydroxylase deficiency (21OHD) is the most common form of CAH, but there are numerous other distinct subtypes as highlighted in Table 1-3 above. Each variant has distinct clinical and biochemical features, and represents a distinct challenge in terms of clinical management. This and the subsection below will concentrate on patients with 21OHD, which accounts for 95% of cases. The frequency of 21OHD in newborns is 1:16,000 (Speiser and White, 2003). 21OHD has traditionally been referred to as 'classic' if the severity of enzyme block is sufficient to result in clinically apparent GC deficiency. 'Non-classic' variants have androgen excess but the milder degree of enzyme deficiency means that there is only compensated GC deficiency. Classic 21OHD is often further subdivided into 'salt-wasting', due to severe mineralocorticoid (MC) deficiency, or 'simple virilising' variants. Classic 21OHD is usually diagnosed at birth, either on routine screening or upon identification of virilisation of external female genitalia, or occasionally on presentation with salt-wasting crisis in patients with severe MC deficiency. Non-classic 21OHD more frequently manifests in adolescence with a PCOS-like phenotype, including non-specific androgen excess and oligomenorrhoea. The distinction between classic and non-classic 21OHD is largely arbitrary, and increasingly viewed as obsolete by experts who regard both variants as a continuous spectrum of the same condition (Auchus and Arlt, 2013).

The key principles of pharmacological management in patients with 21OHD are treatment of adrenal hormone deficiencies and reduction of ACTH drive from the pituitary gland (Joint, 2002). Classic 21OHD requires treatment with oral glucocorticoids and the synthetic mineralocorticoid fludrocortisone. Glucocorticoids will provide physiological replacement in the setting of deficiency, but will simultaneously reduce pituitary ACTH secretion via a negative feedback mechanism. Adults with 21OHD are treated with a variety

of glucocorticoid regimens, and requirements exhibit a large degree of inter-individual variabilility. Hydrocortisone is most commonly prescribed in the paediatric population due to concerns about the effects of prednisolone and dexamethasone on bone density and linear growth (Chakhtoura et al., 2008).

Three daily doses of 5-10mg each are generally sufficient to maintain control. This may be continued in the adolescent and early adult period in the absence of specific indications to change, such as poor compliance or control issues with multiple daily doses. The next step in the event of the latter issues is initiation of prednisolone 0.5-2mg at bedtime. Dexamethasone is the most clinically and biochemically effective downregulator of the HPA axis and ACTH drive in patients with CAH, but patients are more likely to experience complications of glucocorticoid excess than with other steroid preparations. Dexamethasone is usually initiated at dose of 0.25-0.375mg/day, but it should be reserved for patients with complex pathology, such as oligozoospermia due to testicular adrenal rest tumours (TARTs) in men (Mouritsen et al., 2010). It may have a limited role in selected female cases of oligomenorrhoea, androgen excess and subfertility. Mineralocorticoid replacement is required in 21OHD patients with salt-wasting; it is not required in non-classic CAH. Clinical doses of fludrocortisone vary between 100 and 250mcg. Replacement is monitored clinically by measurement of lying and standing blood pressure, and biochemically by measurement of plasma renin levels, and urea and electrolytes (Oelkers et al., 1992). Adult patients are at increased risk of iatrogenic hypertension compared to children and require close clinical surveillance (Falhammar et al., 2007).

Biochemical monitoring in 21OHD is complex and there are misconceptions about the sole use of 17OHP levels as a guide to therapy. Suppression of 17OHP into the normal range with glucocorticoids significantly inreases the risk of over-replacement (Auchus and Arlt,

2013). Calculation of the free androgen index (FAI) via serum T and SHBG levels may be a useful test of biochemical control in 21OHD women (Vermeulen et al., 1999). However measurement of the A:T ratio is a reasonable indirect marker of adrenal versus ovarian androgen generation, which is importance due to the co-existence of PCOS in many of these women. An A:T ratio >4 indicates that most of the testosterone is derived from the adrenal gland, and hence indicative of poor control. In women with 21OHD aiming for conception, follicular phase progesterone levels below 2nmol/l are an important predictor of ovulation. Excessive follicular phase progesterone of adrenal origin has adverse effects on menses and ovulation (Casteras et al., 2009). In men, A:T ratios of >0.5 indicate that a large proportion of T is of adrenal origin; this is due to the high rates of conversion of A to T in the testes by 17βHSD3. An elevated ratio, coupled with reduced semen parameters and suppressed gonadotrophins, in a powerful indicator of poor control in the 21OHD male patient (Claahsen-van der Grinten et al., 2007).

1.4.4.2 Long-term health consequences of monogenic androgen excess in women

CAH and other causes of monogenic androgen excess are lifelong disorders with severe health consequences if untreated or poorly managed. A combination of erratic disease control and, in the case of CAH, over-treatment with glucocorticoids, impacts adversely on a variety of health parameters, including fertility, quality of life and metabolic risk. A recent study of 203 adult patients with CAH in the UK observed that health status is subjectively and objectively very poor in both sexes, but worse in females (Arlt et al., 2010). The prevalence of obesity, dyslipidaemia and insulin resistance was significantly higher in CAH patients than in healthy controls, and quality of life parameters were reduced. Over-zealous glucocorticoid treatment is undoubtedly a major contributor to the health deficiencies described above.

However, 24% of women with non-classic CAH, reflective of those patients who are unlikely to have had lifelong glucocorticoid exposure, had biochemical evidence of insulin resistance compared to 28% of those with the classic subtype. This is supported by a study showing unfavourable metabolic parameters, including increased risk of hypertension, higher stimulated insulin and increased 120-minute OGTT glucose values, in children with non-classic CAH compared to healthy subjects (Williams et al., 2010). This points toward a primary role for androgens in the pathogenesis of metabolic disease in these patients.

1.5 Links between insulin resistance and androgen excess

The relationship between insulin resistance and androgen excess in PCOS has been apparent for many years (Burghen et al., 1980). Burghen and colleagues found strong positive correlations between fasting insulin levels and serum levels of androstenedione and testosterone. As discussed above monogenic IR may lead to profound secondary androgen excess in girls of reproductive age, and these patients often present in adolescence with amenorrhoea and clinical hyperandrogenism. Conversely, girls with monogenic causes of androgen excess such as CAH have evidence of metabolic derangements that cannot be attributed solely to glucocorticoid treatment, while postmenopausal women with hyperandrogenism due to ovarian hyperthecosis have features of the metabolic syndrome and a clustering of cardiovascular risk factors (Barth et al., 1997). In PCOS, women with the so-called ‘non-hyperandrogenic’ Rotterdam phenotype of Anov + PCO (as discussed in section 1.2.1) may be at lowest risk of metabolic dysfunction (Barber et al., 2007, Moghetti et al., 2013). However, the associations are complex, and while hyperandrogenaemic PCOS women appear to have the worst metabolic phenotype, even the normo-androgenaemic subgroup may not be entirely metabolically similar to non-PCOS control women (Di Sarra et al., 2013,

O'Reilly et al., 2014b). The sections below will summarise the *in vivo* and *in vitro* evidence linking insulin resistance and androgen excess.

1.5.1 Evidence for regulation of androgen metabolism by insulin

1.5.1.1 Insulin and the ovary

The observation of androgen excess in genetic syndromes of severe insulin resistance has been discussed above. Hyperinsulinaemia, in the setting of severe insulin resistance at the level of liver, muscle and adipose tissue, may lead to direct stimulation of ovarian and possibly peripheral androgen generation; relative preservation of insulin sensitivity at the level of the ovary and other organs of androgen generation may then facilitate insulin-mediated effects on steroidogenesis. Some authors have postulated that insulin could act via ovarian IGF-1 receptors rather than the INSR to drive androgen production in the setting of pre- or post-receptor defects of insulin action (Willis et al., 1998, Willis and Franks, 1995). However, evidence for this is by no means conclusive, and selective inhibition of the IGF-1 receptor has not been proven to lower insulin-stimulated androgen generation (Nestler et al., 1998). The key role of the INSR in ovulation and ovarian androgen metabolism is further highlighted by a recent study showing reversal of obesity-induced subfertility and androgen excess after INSR deletion (Wu et al., 2014).

Insulin regulates ovarian androgen steroidogenesis through a number of distinct mechanisms (Poretsky et al., 1999). These are summarized in Table 1-4. Insulin receptors are widely expressed in ovarian granulosa, thecal and stromal tissues. *In vitro*, insulin appears to stimulate ovarian androgen generation in humans via upregulation of 17α-hydroxylase activity (Bergh et al., 1993); other possible ovarian steroidogenic targets of insulin action

include 3β-HSD) and P450 side chain cleavage (SCC) enzyme (McGee et al., 1995, McGee et al., 1996). The effects of insulin on ovarian androgen generation are likely to reflect a potentiation of gonadotrophin action at the level of the ovary. In granulosa cells, insulin may increase LH receptor number by acting synergistically with FSH (Adashi et al., 1981). This is supported by *in vivo* data showing that lowering of serum insulin levels via insulin sensitization results in a reduction in circulating LH levels (Nestler and Jakubowicz, 1997). Ovarian growth and cyst formation also appear to be crucially regulated by insulin, possibly via synergism with pituitary gonadotrophins. Circulating insulin levels in women with PCOS correlate with ovarian volume (Pache et al., 1993), and women with genetic syndromes of severe insulin resistance may present with abdominal pain due to enlargement, haemorrhage or torsion of large ovarian cysts (DeClue et al., 1991).

Table 1-4 Summary of effects of insulin on ovarian androgen metabolism

- Direct stimulation of ovarian androgen generation
- Stimulation of steroidogenesis via synergistic action with FSH and LH
- Upregulation of 17α-hydroxylase activity
- Upregulation of LH receptor expression
- Suppression of hepatic SHBG production
- Upregulation of IGF-1 receptor expression
- Potentiation of effect of GnRH on FSH and LH release from pituitary gonadotrophs
- Potentiation of FSH effects on LH receptor recruitment

1.5.1.2 Insulin, circulating androgens and peripheral androgen metabolism

Insulin suppresses hepatic SHBG output, and low circulating levels of the latter are a reliable biochemical marker of underlying insulin resistance. Low SHBG levels result in secondary increases in circulating levels of bioavailable T, leading to a significant rise in the free androgen index (FAI) (Plymate et al., 1988). *In vivo*, the effects of insulin on circulating androgens have been studied extensively. Dunaif *et al* found an increase in A levels during insulin infusion in PCOS women but not in controls (Dunaif and Graf, 1989). Others have found increases in androgen levels (A, T and DHT) during oral glucose tolerance testing in hyperandrogenic and normal women (Smith et al., 1987), although these findings have not been consistently replicated (Tiitinen et al., 1990). No significant effects on circulating androgens were observed in one study during the frequently sampled intravenous glucose tolerance test in PCOS and control women (Falcone et al., 1990).

We have recently published data confirming an inverse relationship between circulating A levels (as measured by LC-MS/MS) and the OGTT-derived insulin sensitivity index (ISI) (O'Reilly et al., 2014b). Nestler *et al* have previously shown that lowering serum insulin levels with metformin therapy in obese women reduced LH concentrations, and also decreased GnRH-stimulated 17OHP levels (Nestler and Jakubowicz, 1996); the same group found similar benefits in lean PCOS women in a separate study (Nestler and Jakubowicz, 1997). Weight loss may also have significant benefits on circulating androgen burden in PCOS (Ernst et al., 2013, Jakubowicz and Nestler, 1997). This may reflect an amelioration of underlying insulin resistance, reduced adiposity or both. The complexity of the associations between hyperandrogenism and metabolic dysfunction in PCOS are highlighted by a recent study from Moghetti *et al*. Metabolic inflexibility, or impaired ability to shift substrate metabolism from predominantly lipid oxidation in fasting states (low serum insulin) towards

predominant glucose oxidation in the fed state, was worst in hyperandrogenic PCOS women (Di Sarra et al., 2013) (Figure 1-13); however, normo-androgenic PCOS women were still metabolically 'inflexible' compared to controls, suggesting the interplay of other poorly-understood factors.

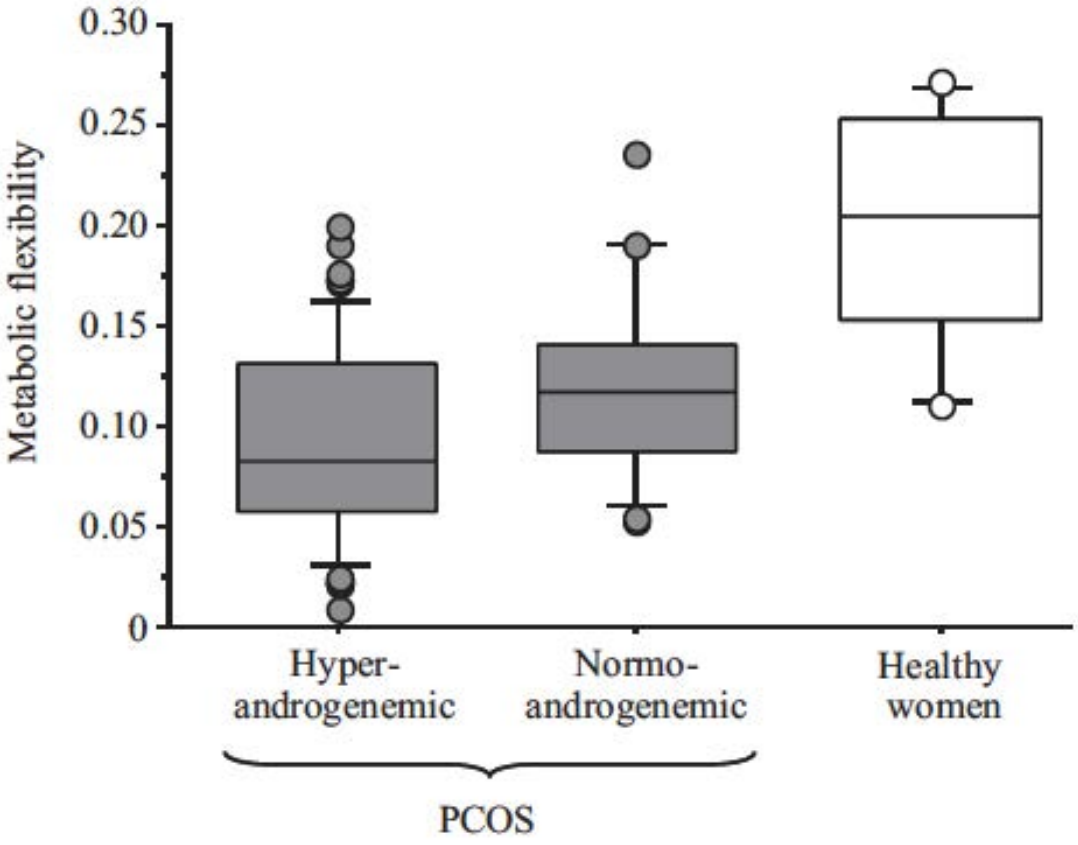

Figure 1-13 Metabolic inflexibility in hyperandrogenic and normo-androgenic PCOS women compared to controls (Di Sarra et al., 2013). Although worst in hyperandrogenic women, normo-androgenic women also had lower metabolic flexibility than controls. Reproduced with permission from the Endocrine Society.

Some studies have shown a strong correlation between urinary indices of systemic 5α-reductase activity and insulin resistance; this association appears to be independent of BMI (Vassiliadi et al., 2009, Tsilchorozidou et al., 2003), and is observed in both lean and obese PCOS subjects (Tomlinson et al., 2008a). The effects of insulin sensitization on peripheral androgen and cortisol metabolism were studied by Glintborg *et al* (Glintborg et al., 2009). The effects of 16 weeks of insulin sensitization with pioglitazone were compared to those of placebo treatment; pioglitazone treatment significantly decreased relative 5αR activity as measured by 5allo-THF/THF ratio. No significant changes were observed in the An/Et ratio or in markers of glucocorticoid metabolism.

1.5.1.3 Insulin and adrenal androgen metabolism

There is also some evidence of interaction between the insulin/glucose axis and adrenocortical dysfunction in PCOS. Circulating DHEAS levels decreased by between 18 and 26% in 305 women treated with the insulin-sensitising agent troglitazone for 20 weeks (Azziz et al., 2003). Similarly, pioglitazone reduced adrenal androgen response to CRH in insulin-resistant women with PCOS (Romualdi et al., 2007); pioglitazone administration for 6 months was separately shown to reduce 17OHP and A responsiveness to ACTH stimulation in obese PCOS patients (Guido et al., 2004). Insulin infusion enhances 17OHP secretion after ACTH administration in hyperandrogenic women (Moghetti et al., 1996). This effect was likely due to upregulation of CYP17A1 activity, with greater proportional upregulation of androgen-generating 17, 20-lyase than 17α-hydroxylase activity. In obese adolescents with PCOS and IGT, insulin sensitization with metformin attenuated the exaggerated pre-treatment adrenal androgen response to ACTH stimulation (Arslanian et al., 2002). *In vitro,* insulin, IGF-1 and IGF-2 stimulated adrenal androgen generation by upregulating ACTH receptor expression in

primary human adrenal reticularis cells (l'Allemand et al., 1996). However McKenna and colleagues found no significant effect of insulin or IGF-1 on adrenal steroidogenesis in isolated guinea-pig adrenal cells (O'Connell et al., 1994).

1.5.2 Evidence linking primary androgen excess with insulin resistance

1.5.2.1 Premature adrenarche and metabolic disease

There is strong evidence that early androgen exposure is causally linked to the development of metabolic dysfunction in women. Premature adrenarche (PA) and premature pubarche (PP) are risk factors for subsequent development of PCOS, insulin resistance and early onset of metabolic disturbance. American-Hispanic girls who presented with signs of PP were noted to be insulin resistant (Oppenheimer et al., 1995). Ibanez and colleagues have looked extensively at the association between insulin resistance and PP. Impaired insulin sensitivity appears to be a consistent finding in multiple studies (Ibanez et al., 1996, Ibanez et al., 1997); dyslipidaemia has also been documented in Catalonian PP girls, coupled with other adverse cardiovascular markers such as increased waist-to-hip ratio and central fat accumulation (Ibanez et al., 1998, Ibanez et al., 2003). Early androgen exposure also has potential associations with increased blood pressure (Teixeira et al., 2004). The potential role of androgens in fat distribution and adipose tissue function will be discussed in detail in section 1.7.

PP has a strong association with development of PCOS in subsequent years. PCOS was identified in 45% of Catalonian girls with a history of PP in one study (Ibanez et al., 1993). PP has links to both ovarian and adrenal hyperandrogenism; girls with a history of PP had exaggerated androgen responses to both GnRH analogues and ACTH stimulation, and

responses to both correlated with serum insulin levels (Ibanez et al., 1996). Interestingly, PA and androgen excess due to sulfation defects in families harboring PAPPS2 mutations is strongly associated with metabolic disturbance and PCOS (Oostdijk et al., 2015, Noordam et al., 2009). It is also tantalizing that heterozygosity for the culprit mutation has been linked with PCOS and ovulatory dysfunction in apparently unaffected female family members in both studies. This provides strong evidence for a causal role of androgens in the development of metabolic disease and polycystic ovaries. There is a need for carefully controlled prospective longitudinal studies to unravel this association between early onset androgen excess and metabolic disturbance.

1.5.2.2 Prenatal androgen excess and metabolic disturbance

Animal data highlights a key role for androgens in prenatal fetal metabolic programming. Studies have consistently identified the consequences of fetal androgen exposure *in utero* on early postnatal and long-term metabolic health of offspring. Prenatal androgenisation in multiple animal models has been shown to confer metabolic, reproductive and endocrine disturbances similar to PCOS (Walters et al., 2012, Padmanabhan and Veiga-Lopez, 2013). Mice treated prenatally with DHT developed ovarian cysts, ovulatory dysfunction and increased adipocyte size (Caldwell et al., 2015). AR knock-out (ARKO) mice in the same study were protected from adipocyte hypertrophy. This highlights the key role played by androgens and adipose tissue in metabolic disturbances in PCOS. Prenatal treatment of female mice with 250µg DHT pellets between days 16 and 18 of pregnancy induced reproductive-metabolic abnormalities in female offspring (Roland et al., 2010). Metabolic disturbances included increased visceral adipocyte size, elevated fasting glucose and impaired glucose tolerance. In rats, prenatal exposure to a single dose of testosterone

(5mg at gestational day 20) resulted in increased weight and insulin resistance in female offspring, with differences compared to controls evident at days 30, 45, 60 and in adulthood (Tehrani et al., 2014). Similar findings have been reported in monkey, sheep and rat models of PCOS (Abbott et al., 2005, Yan et al., 2013, Padmanabhan et al., 2010).

It is much more difficult to study human models of prenatal androgen exposure. However, as in animals, the intrauterine environment is likely to play a critical role in fetal metabolic programming, growth and development. A prospective study of infants born to women with PCOS highlighted an increased prevalence of small for gestational age (SGA) infants compared to infants born to non-PCOS women (Sir-Petermann et al., 2005). Dunaif and colleagues found lower umbilical cord levels of serum androstenedione in infants of women with PCOS compared to controls (Anderson et al., 2010); this finding does not support the hypothesis that intrauterine androgen excess contributes to the development of PCOS. However, another study found that serum levels of anti-mullerian hormone (AMH) and ovarian androgens were reduced in infancy in daughters of PCOS women treated with metformin throughout pregnancy compared to metformin-naïve PCOS controls (Crisosto et al., 2012). This provides a fascinating insight into the prenatal role of insulin and androgens in fetal reproductive-metabolic development.

1.5.2.3 In vivo evidence linking primary hyperandrogenism with metabolic disturbance

Insulin resistance is highly prevalent in female patients with CAH. However as discussed above, the confounding effects of treatment with glucocorticoids in patients with classic CAH means that women with non-classic CAH represent a pharmacologically ‘cleaner’ group in whom to define the effects of androgen excess on metabolism; most patients are diagnosed in adulthood, and have a relatively short history of glucocorticoid

treatment (if any at all). Speiser found evidence of reduced insulin sensitivity in 6 females with non-classic CAH compared to age and BMI-matched controls (Speiser et al., 1992); none of the CAH patients were receiving glucocorticoid treatment at the time. Other studies have also found a close correlation between hyperinsulinaemia and androgen excess in newly-diagnosed untreated adult female CAH patients compared to healthy controls (Saygili et al., 2005, Zhang et al., 2010).

Children of both sexes diagnosed with non-classical CAH may also at risk of metabolic disturbance even by the end of the first decade of life (Williams et al., 2010). Insulin resistance and reduced lean body mass in these young non-classical CAH cohort are likely to indicate the adverse metabolic effects of early androgen exposure rather than long-term glucocorticoid treatment. In one study, adolescents with non-classical CAH also already showed early signs of derangements in vascular intima media thickness, indicating a possible role of androgens in the pathogenesis of cardiovascular disease (Wasniewska et al., 2013).

There is also some evidence linking androgen treatment with metabolic disturbance in patients undergoing female-to-male gender reassignment, although data have shown conflicting results. T dosages administered to female-to-male transsexuals are similar to those administered to men with hypogonadism. One study showed that glucose utilization (a marker of peripheral insulin sensitivity) on hyperinsulinaemic euglycaemic clamp was reduced in female-to-male gender reassignment patients after 4 months of T treatment (Polderman et al., 1994). Baseline indices of insulin sensitivity were unaffected by 12 months of T therapy in a separate study (Elbers et al., 2003). However, methyltestosterone administration to healthy women significantly reduced whole-body glucose uptake during high-dose insulin infusion in another clamp study (Diamond et al., 1998). T treatment may also result in a decline in serum levels of HDL cholesterol (Asscheman et al., 1994). Further longitudinal studies are required

to tease out these associations. It is also notable that polycystic ovaries are a universal finding after long-term androgen exposure (>18 months) in such patients (Gooren and Giltay, 2008). Histological findings include thecal hyperplasia, luteinization and stromal hyperplasia.

1.5.2.4 Direct effects of androgens on tissue-specific insulin sensitivity

Relatively few human or animal studies have directly examined the effects of androgens on insulin and glucose metabolism in target tissues of insulin action. T treatment for 48 hours significantly reduced insulin-stimulated glucose uptake in differentiated subcutaneous adipocytes of premenopausal women in one small human study (Corbould, 2007) (Figure 1-14). The observed defects were independent of PI3K activation and were ameliorated by androgen receptor blockade. Studies of androgen-induced effects on skeletal muscle insulin sensitivity are also sparse in humans. One study found that T exposure *ex vivo* significantly increased androgen receptor and aromatase gene expression in myotubes from both PCOS and control women, but exerted no effects on insulin-mediated glucose disposal (Eriksen et al., 2014). In animals, T treatment of female rats for between 8 and 12 weeks caused hyperinsulinaemia in both intact and ovariectomised animals (Holmang et al., 1990); in the ovariectomised group, T treatment was associated with a 50% reduction in insulin-stimulated glucose uptake into skeletal muscle.

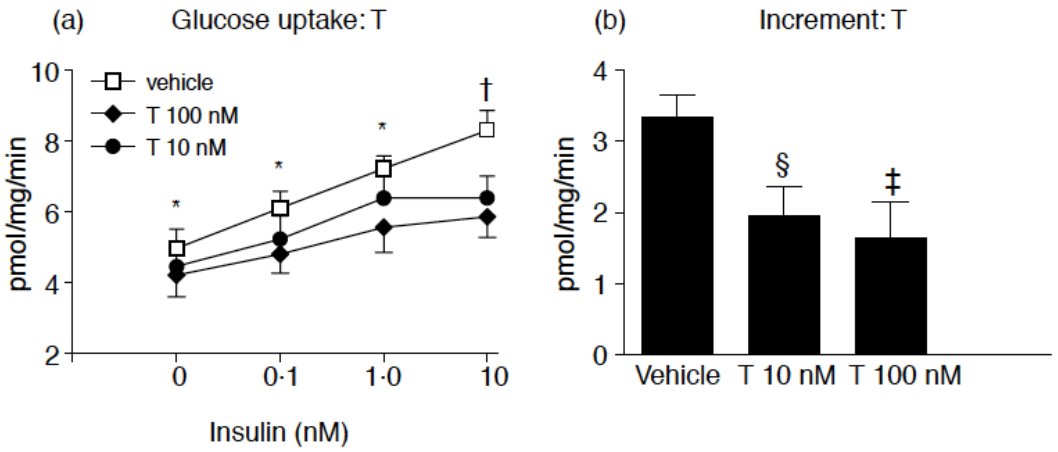

Figure 1-14 Effects of testosterone on insulin-stimulated glucose uptake in differentiated female subcutaneous adipocytes. Testosterone reduced glucose uptake in a dose-dependent fashion; effects were reversed after AR blockade (not shown) (Corbould, 2007). Reproduced with permission from BioScientifica Ltd.

1.6 Adipose tissue

1.6.1 Adipose tissue embryology and morphology

Human adipose tissue (fat) development begins *in utero* during the second trimester of pregnancy. In the fetus, adipose tissue develops from the embryonic mesenchyme (Greenwood and Johnson, 1977). Adipose tissue consists of lipid-containing adipocytes, and a stromal-vascular compartment containing fibroblast-like preadipocytes, supported by a matrix of collagen fibres (Poissonnet et al., 1984). Fetal fat includes both white (WAT) and brown adipose tissue (BAT); the latter is multi-locular, localized around the neck and interscapular region, and a crucial player in fetal thermoregulation. Most BAT is lost in early childhood, although small depots persist into adulthood, located in the cervical, supraclavicular, paravertebral, mediastinal, para-aortic and supra-renal regions (Nedergaard et al., 2007). BAT appears to correlate negatively with adverse metabolic risk and obesity, and is positively

associated with increased energy expenditure and, by inference, weight loss (Nedergaard and Cannon, 2013). The sections below will focus on WAT biology.

WAT is organised in various distinct anatomical locations, notably in the subcutaneous (SC) fat depot and the intra-abdominal fat depot. These depots are considered to be metabolically distinct, and intra-abdominal fat accumulation is a potent risk factor and surrogate marker for metabolic disease, including insulin resistance, dyslipidaemia and NAFLD (Kranendonk et al., 2015). In adulthood, SC WAT exhibits a sexually dimorphic pattern of distribution; in females, adipose tissue is preferentially accumulated in the gluteofemoral region, while men predominantly accumulate fat in the abdominal (central) region (Varlamov et al., 2014). Intra-abdominal fat is distributed across the omental, retroperitoneal and peri-renal depots. A substantial portion of intra-abdominal WAT is also located around the intestines, liver and other intra-abdominal organs, and is referred to visceral fat (Gesta et al., 2007). The key processes of adipose tissue expansion are hyperplasia and hypertrophy. Hyperplasia (also called adipogenesis) refers to an increase in fat cell number; this process is driven by (i) preadipocyte proliferation and (ii) adipocyte differentiation. Fat cell hypertrophy is an increase in adipocyte size as a consequence of intracellular lipid accumulation. These processes are tightly regulated by a complex system of intracrine and paracrine factors, and are crucial mediators of metabolic health in humans.

1.6.2 Adipose tissue proliferation

Mesenchymal stem cells (MSCs) are multipotent, with potential to differentiate into a variety of different tissues, including bone, adipose and muscle. After commitment to the adipocyte lineage, MSCs are transformed into fibroblast-like preadipocytes (Figure 1-15). The preadipocyte pool is located in the stromal-vascular compartment of mature adipose tissue.

Clonal expansion of the preadipocyte pool appears to occur in three stages; firstly with MSC commitment to the preadipocyte lineage, secondly as preadipocyte proliferation, and lastly via another later round of proliferation after the process of differentiation has begun. The potential to generate new fat cells from preadipocytes persists throughout life, although the number of cells capable of differentiating reduces significantly with age (Bjorntorp, 1979). Adipogenesis is tightly regulated by a complex network of endocrine, paracrine and nutritional factors.

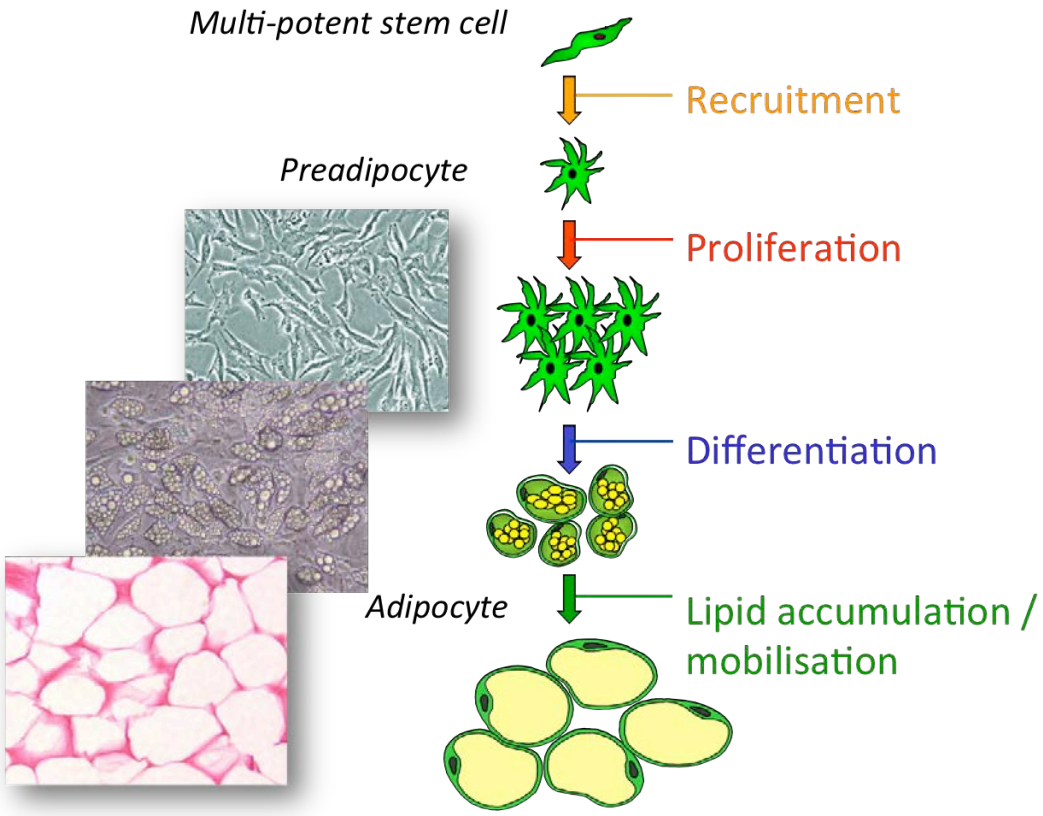

Figure 1-15 The process of adipogenesis, from MSC commitment to the preadipocyte lineage, followed by preadipocyte proliferation and differentiation into mature adipocytes, leading to adipose hyperplasia. The processes of lipid accumulation and mobilization are delicately balanced; increased accumulation results in adipocyte hypertrophy.

Preadipocytes isolated *in vitro* from the stromal-vascular compartment of adipose tissue may be subdivided into those already committed to differentiation, and those comprising a persistently replicating subgroup of fibroblast-like cells (Entenmann and Hauner, 1996). Adipocytes themselves are capable of stimulating preadipocyte proliferation in a paracrine fashion, and this capacity appears to be increased in obese individuals (Considine et al., 1996). Furthermore, specific WAT depots may exhibit greater preadipocyte proliferation potential than others. Preadipocytes from visceral fat depots produce greater quantities of basic fibroblast growth factor (bFGF) compared to their SC WAT counterparts, thereby increasing proliferation potential (Teichert-Kuliszewska et al., 1992). This may explain the increased expansion potential observed in omental and visceral adipose tissue.

1.6.3 Adipose tissue differentiation

Preadipocytes differentiate into mature adipocytes via a meticulously regulated process, which is modulated by both stimulatory and inhibitory signals (Farmer, 2006). The key step in the adipocyte differentiation process appears to be preadipocyte growth arrest; cell confluence or cell-to-cell contacts appear to be of less importance (Reichert and Eick, 1999). Differentiation can be divided into two stages. In the initial step (commitment), growth arrest takes place, and morphological changes are observed, with altered cytoskeleton and extracellular matrix (ECM) (Amri et al., 1986). The net effect is a change in appearance of the stellate-shaped preadipocytes into a more spherical cellular structure. Early markers of differentiation include expression of lipoprotein lipase (LPL) (Grimaldi et al., 1979). The second phase of adipocyte differentiation is associated with expression of late markers such as glycerol-3phosphate dehydrogenase (G3PDH); maturing adipocytes begin to accumulate

multiple small lipid droplets, which ultimately coalesce into a single large unilocular lipid droplet upon maturity.

In the initial phase of differentiation, cytoskeletal changes precede the expression of adipocyte transcription factors (Spiegelman and Farmer, 1982). Cytoskeletal changes ultimately producing the spherical-shaped mature adipocyte may therefore not depend on intracellular lipid accumulation. Initiation of differentiation depends on a shift in the delicate balance between the multiple stimulatory and inhibitory signals that regulate adipogenic transcription factors. Cytoskeletal and ECM changes are associated with specific changes in expression of key genes; for example fibroblast-expressed type I and type III collagen are suppressed, while α_2-collagen type IV gene expression is significantly increased (Ailhaud et al., 1989). Similarly, pre-adipocyte factor 1 (pref-1) is a transmembrane protein which maintains the preadipocyte phenotype and suppresses differentiation by inhibiting peroxisome proliferator activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein (C/EBP)α expression (Berry et al., 2012, Smas and Sul, 1997); pref-1 gene expression is entirely abolished across differentiation.

PPARγ is the principal transcriptional regulator of adipogenesis, and its expression is reliant on the C/EBP family of transcription factors. Adipogenesis is completely inhibited in PPARγ-knockout mice (Kubota et al., 1999); in humans, activating missense mutations in the PPARγ2 splice variant result in accelerated differentiation and severe obesity (Ristow et al., 1998). Expression of PPARγ and C/EBPα is high throughout differentiation, and remains high in the mature adipocyte. Another crucial player in adipocyte differentiation is the sterol-regulatory element binding protein-1 (SREBP) subclass of transcription factors (Sewter et al., 2002). SREBP-1c acts directly to enhance the transcriptional activity of PPARγ (Fajas et al., 1999). It also plays a major role in fatty acid metabolism.

In the late stages of preadipocyte differentiation, mRNA and protein expression of enzymes involved in key metabolic functions of the adipocyte are significantly upregulated. Induction of INSR and GLUT-4 gene expression result in increased insulin sensitivity (Serrano et al., 2005). Mirroring this, there is a massive increase in expression of genes involved in lipid metabolism; these enzymes are integral to lipid flux in the adipocyte, and control such processes as *de novo* lipogenesis, FFA uptake, β-oxidation and lipolysis. These enzymes include fatty acid synthase (FAS), Acetyl Coenzyme A Carboxylase (ACC), LPL and hormone sensitive lipase (HSL). Expression of G3PDH is also a marker of terminal adipocyte differentiation. Lipid metabolism is discussed in detail in section 1.6.4.

1.6.3.1 Hormonal regulation of adipogenesis

The process of adipogenesis is also governed by a number of important hormones. Insulin is a key player in adipocyte differentiation, increasing the proportion of differentiating preadipocytes, as well as playing a major role in adipocyte lipid accumulation (see below). INSR expression is low in the early stages of adipogenesis; insulin action is therefore mediated via the IGF-1 receptor (Smith et al., 1988). In 3T3-L1 cells, INSR expression increases from 3,000-20,000 receptors per cell early in adipogenesis to 170,000-250,000 receptors in the mature adipocyte (Turnbow et al., 1994). INSR activation leads to rapid phosphorylation of IRS-1, ultimately leading to stimulation of the PI3K and MAP kinase pathways. Depot-specific differences in both INSR and IRS-1 expression have been observed in obese females, with increased expression, binding and activation observed in femoral compared to omental adipocytes (Bolinder et al., 1983). Thyroid hormone also stimulates adipocyte differentiation (Flores-Delgado et al., 1987). Tri-iodothyronine (T3) modulates both proliferation and differentiation to adipocytes (Darimont et al., 1993). Mice with mutated

thyroid hormone-α1 receptor (TRα1) have reduced WAT mass across a variety of depots; this appears to be due to repression of PPARγ and C/EBPα expression, resulting in impaired adipogenesis (Ying et al., 2007).

Adipogenesis is also potently induced by glucocorticoids in the presence of insulin (Hauner et al., 1987). Patients with Cushing's syndrome due to endogenous glucocorticoid excess, or with iatrogenic disease due to exogenous glucocorticoid treatment, have increased visceral adiposity. Glucocorticoids stimulate transcription of a number of important adipogenic genes, including G3PDH and LPL (Hauner et al., 1989). *In vitro*, synthetic glucocorticoids are an essential tool in primary adipocyte culture and in fat cell lines, stimulating differentiation from preadipocytes to adipocytes. Key pro-adipogenic transcription factors such as C/EBPs and PPARγ, are upregulated by glucocorticoids (Vidal-Puig et al., 1997); conversely, the anti-adipogenic factor pref-1 is transcriptionally repressed by the potent synthetic glucocorticoid dexamethasone (Smas et al., 1999). Glucocorticoid effects on adipogenesis appear to be mediated via the glucocorticoid receptor (GR) (Ottosson et al., 1995).

1.6.4 Adipocyte lipid metabolism

Adipocytes are, first and foremost, lipid-storing cells. Lipid flux refers to the delicate balance between lipid accumulation, mobilization and utilization in the adipocyte (Figure 1-16). When this balance is tipped in favour of lipid accumulation over lipid breakdown, the net result is adipocyte hypertrophy. Lipid flux is tightly regulated as part of a complex metabolic pathway, and is dependent on factors such as nutritional status, caloric intake, insulin sensitivity and various lipogenic and lipolytic stimuli. The key processes in adipocyte lipid metabolism are summarized in the sections below.

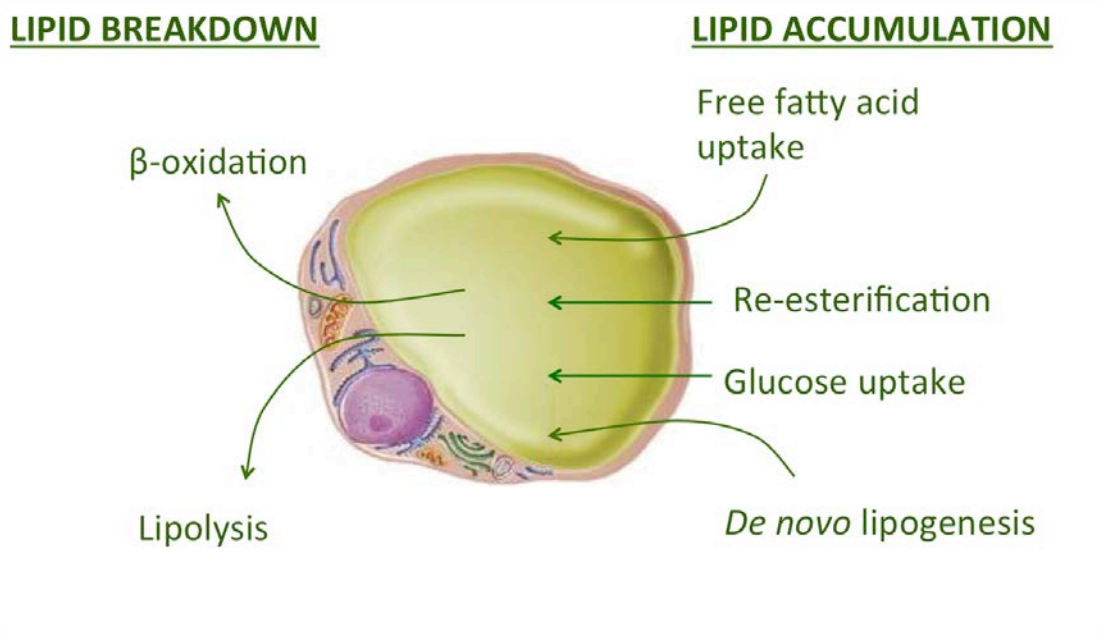

Figure 1-16 Lipid flux in the adipocyte. Lipid metabolism is a balance between lipid accumulation on the right and lipid breakdown on the left. The main processes driving lipid accumulation are free fatty acid (FFA) uptake, re-esterification of FFAs with glycerol to form triacylglycerol (TAG), generation of glycerol from glucose (glycolysis), and de novo synthesis of TAG. The main pathways of lipid mobilization are lipolysis, the end products of which are FFAs and glycerol, and β-oxidation, by which FFAs are broken down to generate energy in the mitochondrion.

1.6.4.1 Adipose lipogenesis

Adipose lipogenesis (Figure 1-17) occurs as a consequence of either re-esterification of dietary-derived FFAs with glycerol to form TAG, or generation of TAG within the adipocyte from *de novo* fatty acid synthesis (Gathercole et al., 2011). *De novo* fatty acid synthesis may account for up to 20% of adipocyte triglyceride turnover (Strawford et al., 2004). Acetyl CoA is produced in the adipocyte mitochondria and is combined with oxaloacetate by citrate synthase to form citrate. Citrate moves from the mitochondria to the

cytosol, where citrate lyase releases oxaloacetate and acetyl coA. The latter is then carboxylated to malonyl CoA in an ATP-dependent reaction by ACC1. This is the first and rate-limiting step in fatty acid synthesis. Malonyl CoA is subsequently converted by a multi-step reaction to the fatty acid palmitate by fatty acid synthase (FAS) (Ruderman et al., 2003). TAG is then formed by the esterification of three fatty acid chains to one glycerol-3-phosphate molecule. In the fed state, when insulin and glucose are abundant, glycerol-3-phosphate is derived from glucose by G3PDH via glycolysis. In the fasted state, glycerol-3-phosphate may be generated by glyceroneogenesis.

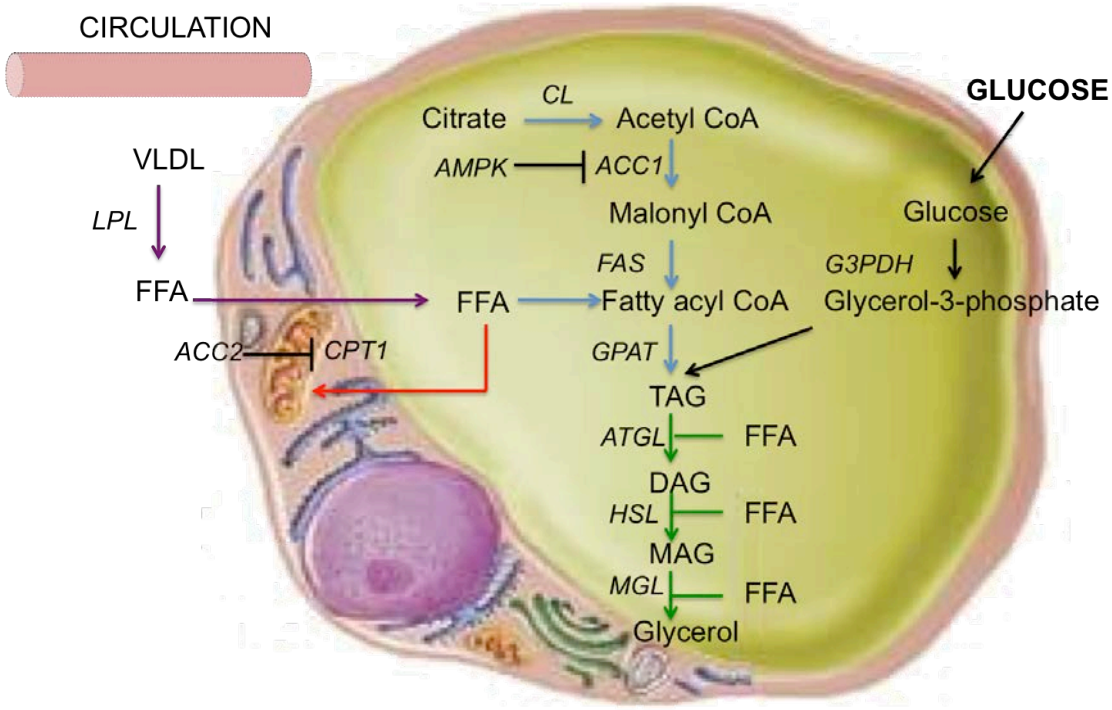

Figure 1-17 Adipocyte lipid metabolism. The balance between lipid uptake (purple arrows), lipogenesis (blue arrows) and lipolysis (green arrows) is tightly regulated. Extracellular glucose is also be taken up by the adipocyte to generate glycerol-3-phosphate via the glycolytic pathway (black arrows), and free fatty acids are oxidized in the mitochondrion to generate energy (red arrow). ACC1, acetyl coA carboxylase I; ACC2, acetyl coA carboxylase 2; AMPK, AMP-kinase; ATGL, adipose triglyceride lipase; CPT1, carnitine palmitoyl transferase I; CL, citrate lyase; DAG, diacylglycerol; FFA, free fatty acid; GPAT, glycerol-3-phosphate acyltransferase; G3PDH, glycerol-3-phosphate dehydrogenase; HSL, hormone sensitive lipase; LPL, lipoprotein lipase. MGL, monoglyceride lipase; VLDL, very-low density lipoprotein.

ACC1 is highly post-transcriptionally regulated. As well as its role as a precursor of acetyl CoA, cytosolic citrate is an allosteric activator of ACC. ACC1 mRNA expression is increased by glucose and insulin in physiological situations of fuel surplus, eg the ‘fed’ state. The regulatory effects of insulin on ACC1 are mediated by transcription factors such as SREBP1c and FOXO (Zhang et al., 2003). Protein levels of ACC1 are also susceptible to

proteasomal degradation and ubuiqitination. Tribbles 3 (TRB3) is a pseudokinase which targets ACC for ubuiqitination (Qi et al., 2006); levels of TRB3 increase in the fasted state (Du et al., 2003), thereby reducing ACC protein levels and encouraging lipid mobilization rather than accumulation in states of reduced cellular fuel availability. AMP-Activated Protein Kinase (AMPK) is a crucial inhibitor of ACC1 activity (Ruderman et al., 2003), and is also activated by decreases in the energy state of the cell. This low-fuel state is indicated by increases in the AMP/ATP ratio. AMPK phosphorylates ACC1 at Ser79, 1200 and 1215, negatively regulating its activity. Additionally, activated AMPK inhibits allosteric activation of ACC by citrate.

Insulin and glucose can reverse the negative regulation of ACC by AMPK in the fed state, both by increasing cytosolic citrate concentrations and reducing AMPK activity (Itani et al., 2003). Insulin and glucose increase expression of G3PDH, thereby increasing glycerol-3-phosphate synthesis, with a secondary increase in adipocyte TAG generation (Moustaid et al., 1996). Insulin is also a potent stimulator of FAS mRNA expression and activity in adipose tissue, leading to increased generation of palmitate from malonyl CoA.

1.6.4.2 Lipolysis

In the fasted state, TAG stored in WAT is mobilized to produce FFAs, thereby acting as the body's major energy reserve. TAG is hydrolysed in the fasted state, releasing FFAs and glycerol; this is possible in the setting of low glucose and insulin levels (Masoodi et al., 2015). The main enzymes facilitating intra-adipocyte lipolysis are adipose triglyceride lipase (ATGL), HSL and monoglyceride lipase (MGL) (Figure 1-17). ATGL catalyses the initial step in TAG hydrolysis and is the rate-limiting step (Haemmerle et al., 2006); HSL catalyses the hydrolysis of TAG into diacylglycerols (DAG) and then monoacylglycerols (MAG);

MGL removes the final FFA group to release glycerol (Zechner et al., 2012). The lipolytic process is tightly regulated. The enzymes involved in lipolysis are located in droplets called adiposomes, located within the adipocyte, and can interact with other cellular structures such as mitochondria, peroxisomes and the endoplasmic reticulum via these adiposomes.

Lipolysis is stimulated via activation of adipose β-adrenoreceptors; this leads to increased intracellular cAMP levels, which in turn activates protein kinase A (PKA). PKA then phosphorylates HSL, facilitating translocation of the enzyme from the cytosol to the surface of the lipid droplet (Clifford et al., 1997). PKA also phosphorylates proteins called perilipins. In the non-phosphorylated state, perilipins form a protective barrier around the adipocyte lipid droplet, protecting it from the lipolytic action of HSL (Brasaemle et al., 2000). After phosphorylation by PKA, perilipins are removed from the droplet surface and HSL can hydrolyse its lipid substrate. Other crucial regulators of lipolysis include insulin and glucocorticoids. Insulin lowers intracellular levels of cAMP, thereby preventing PKA-induced phosphorylation of HSL, thereby suppressing lipolysis. Glucocorticoids are potent drivers of lipolysis through effects on HSL and ATGL (Zammit, 1999, Villena et al., 2004).

1.6.4.3 Glyceroneogenesis and re-esterification

FFAs are released from adipocytes as part of the lipolytic process, serving as fuel for extra-adipose body tissues. Serum FFA levels play a crucial role in metabolic disease, and levels are consistently elevated in obesity, representing a ‘spill-over’ process in the setting of excess adiposity (Carpentier et al., 2002). Increased circulating FFAs may have consequences for whole-body insulin sensitivity, with effects on glucose metabolism in liver, muscle and peripheral tissues (Shulman, 2014, Thompson et al., 2000). FFAs exert effects on insulin action due to a number of inhibitory effects at crucial points in the insulin signaling pathway.

These include reduced phosphorylation and activation of akt, IRS-1 and PI3K (Kim et al., 2002). This suggests that FFAs may play a prominent role in the link between obesity and insulin resistance. Increased insulin resistance then leads to an inability to adequately suppress adipose lipolysis in the fed state, leading to a vicious circle of lipolysis, FFA release and impaired insulin sensitivity.

Re-esterification of some FFAs into TAG may occur in the fasted state, even when lipolysis predominantes (Lisch et al., 1973). Glycerol-3-phosphate is required for this process of re-esterification, but it cannot be generated from glucose by the glycolytic action of G3PDH in the fasted state due to reduced glucose availability. Adipocytes also cannot recycle glycerol after lipolysis to fuel this process, although this is possible in the liver due to expression of the enzyme glycerol kinase. Glyceroneogenesis refers to the process whereby adipocytes generate glycerol-3-phosphate via dihydroxyacetone phosphate (DHAP) from lactate, pyruvate and amino acids (Ballard and Hanson, 1967). A crucial enzyme in this pathway is phosphoenolpyruvate carboxykinase (PEPCK), which converts oxaloacetate into phosphoenolpyruvate and carbon dioxide. Inhibition of this enzyme by insulin underpins the latter's role in suppression of glyceroneogenesis (Franckhauser et al., 2002). This means that, in the postprandial state, insulin ensures that the glycerol-3-phosphate required for *de novo* lipogenesis is derived from the glycolytic pathway rather than from glyceroneogenesis. In conditions of absolute insulin deficiency, such as T1DM, failure of this controlled system means that glycerol (and body fuel) is generated from the breakdown of lactate, pyruvate and amino acids, leading to generation of ketones and potential metabolic acidosis.

1.6.4.4 FFA uptake

Adipocytes take up dietary-derived FFAs, as well as those released by the liver. Together with *de novo* lipogenesis, this represents the second major source of FFAs for TAG synthesis by the adipocyte. Dietary TAG is digested by pancreatic lipase in the upper segment of the jejunum, releasing glycerol and FFAs. FFAs are taken up from the intestinal lumen by the enterocytes, and packaged into chylomicrons. These are large, spherical, TAG-rich particles, composed of an inner lipoprotein core of TAG, cholesteryl esters and fat–soluble vitamins; the surface consists a hydrophilic coating of phospholipids and apoplipoproteins (Abumrad and Davidson, 2012). Chylomicrons are exported from the enterocyte via the ER into the lymphatic system, from where they enter the circulation and target tissues such as muscle and heart for fuel, or adipose tissue for storage. TAG generated in the liver due to hepatic lipogenesis is packaged as very-low density lipoprotein (VLDL), which, like chylomicrons, contains TAG, cholesterol esters, phospholipids and apolipoproteins (Mayes, 1970).

TAG transported in circulation in VLDL and chylomicrons cannot cross the adipocyte cell membrane, and must be hydrolysed to release FFA and glycerol. This reaction is catalyzed by lipoprotein lipase (LPL) (Li et al., 2014). LPL is expressed and secreted by adipocytes, and after synthesis is translocated to the vascular lumen of adipose blood vessels. FFA released by LPL may enter the adipocyte by simple diffusion, or via specific membrane transporters such as fatty acid transporter protein (FATP) (Abumrad et al., 1999), fatty acid translocase (FAT) (Hanssler et al., 1992) and caveolin-1 (Trigatti et al., 1999). After uptake into the adipocyte, FFAs may be esterified to acyl CoA by acyl CoA synthetase (ACS) (Hames et al., 2015), thereby preventing FFA diffusion back out of the adipocyte. FFAs are

bound to key fatty acid binding proteins, such as Fatty Acid Binding Protein 4 (FABP4) in the adipocyte cytoplasm, which facilitate their transport to important intracellular targets.

In its role as a promoter of lipid storage and accumulation, insulin drives FFA uptake into adipocytes by increasing adipocyte LPL expression and endoluminal activity (Rosato et al., 1997). PPARγ is a potent transcriptional activator of LPL expression, and expression of the former is upregulated by insulin (Vidal-Puig et al., 1996). Additionally, insulin stimulates adipocyte ACS transcription, favouring conversion of FFAs to acyl CoA and thereby preventing diffusion of FFAs out of the adipocyte (Kansara et al., 1996).

1.6.4.1 β-oxidation

β-oxidation is the process by which fatty acyl CoAs are broken down in the mitochondria to generate NADH, FADH2 and acetyl CoA. This occurs in the fasted state when fuel availability is reduced. After feeding, insulin suppresses further β-oxidation. Acetyl CoA generated via this process may then enter the Krebs cycle to produce ATP. The rate-limiting step in β-oxidation is the transport of fatty acyl CoA into the mitochondria by carnitine palmitoyltransferase I (CPT1) (Frayn, 2010). CPT1 is located in the outer mitochondria membrane, where it generates acyl carnitine by transfer of the fatty acid chain to a carnitine molecular. Acyl carnitine enters the inter-membrane space, and is transported across the inner membrane into the mitochondrion by carnitine-acylcarnitine translocase (CAT). Inside the mitochondria, carnitine palmitoyl transferase 2 (CPT2) regenerates fatty acyl CoA, which then enters a series of reactions to generate FADH2, NADH and acetyl CoA. FADH2 and NADH generate two and three ATP molecules respectively, while acetyl CoA enters the Kreb's cycle, ultimately generating 12 ATP molecules.

ACC2 is the major negative regulator of β-oxidation; it inhibits CPT1 action in the outer mitochondrial membrane by generating malonyl CoA. In the fed state, insulin stimulates ACC transcription, thereby suppressing β-oxidation and promoting lipid storage. ACC2, like ACC1, is itself negatively regulated by AMPK, which promotes β-oxidation and lipid mobilization.

1.7 Adipose tissue and androgens

Adipose tissue is a crucial target of androgen action in humans and primates (Varlamov et al., 2012), and AR expression, binding and activation in adipose tissue are well-characterised (Deslypere et al., 1985, Feher et al., 1976). Androgen action in adipose tissue may in part explain sexual dimorphism in patterns of body fat distribution (Nedungadi and Clegg, 2009, Pond, 1992). Men preferentially accumulate fat in an abdominal or android distribution, and have a significantly greater proportion of intra-abdominal or visceral fat compared to females (Lemieux et al., 1993). In contrast, women typically have a greater proportion of peripheral and subcutaneous fat, particularly in the gluteofemoral depot (Wells, 2007). Abdominal, and particularly visceral, fat is a critical mediator of metabolic dysfunction and is associated with dyslipidaemia and metabolic syndrome, thereby conferring an increased risk of T2DM and cardiovascular disease (Despres and Lemieux, 2006, Despres, 2006, Smith et al., 2001). This male predisposition to accumulate visceral fat is a major determinant of increased metabolic risk and IR in men compared to women (Frias et al., 2001, Yki-Jarvinen, 1984, Soeters et al., 2007). In addition to its role as a target organ of androgen action, adipose tissue is also increasingly recognized as an important modulator of pre-receptor metabolism, regulating its exposure to androgens in an autocrine manner (Quinkler et al., 2004, Blouin et al., 2003).

However the precise molecular biology and metabolism of androgens in adipose tissue is complex, with local exposure regulated by a large network of activating and inactivating enzymes (Labrie, 1991). Delineation of specific pathways has proven divisive and many data have shown conflicting results. This field is further complicated by the sex-specific and depot-specific behaviour of steroids in adipose tissue (Blouin et al., 2005, Blanchette et al., 2005, Bujalska et al., 2002). It is clear for many years, however, that adipose tissue is capable of both active androgen synthesis and inactivation (Bolt and Gobel, 1972), and that androgens themselves modulate adipose tissue function (Hansen et al., 1980). Androgen metabolism and action in adipose tissue may be differentially regulated in men and women, and in different body fat depots.

1.7.1 Effects of androgens on adipose tissue function

As discussed above, the key processes of adipose tissue expansion are hyperplasia and hypertrophy. Hyperplasia (or adipogenesis) refers to an increase in fat cell number and is determined by (i) preadipocyte proliferation and (ii) adipocyte differentiation. Fat cell hypertrophy implies an increase in adipocyte size due to intracellular lipid accumulation. Adipogenesis and lipid flux may both be influenced by androgen action, thereby controlling regional fat mass and adipose tissue function. Some of these processes are discussed below and summarized in Table 1-5 below.

In men, central fat accumulation has an inverse relationship with circulating T levels in both cross-sectional (Pasquali et al., 1991) and longitudinal studies (Gapstur et al., 2002). This may be driven by reduced SHBG in the context of abdominal obesity and insulin resistance; the same studies show an inverse association between waist circumference and SHBG levels. Visceral fat accumulation on computed tomography (CT) scanning has a strong

negative correlation with serum SHBG in several studies (Couillard et al., 2000, Garaulet et al., 2000). The relationship between circulating serum sex steroids in women is more complex; women with PCOS and associated hyperandrogenism may have concomitant abdominal obesity (Mongraw-Chaffin et al., 2015).

1.7.1.1 Effects of androgens on preadipocyte proliferation and adipogenesis

The association between androgens and body fat distribution led to the hypothesis that the former may exert a direct effect on adipocyte differentiation (Seidell et al., 1990, Lacasa et al., 1995). Androgens have been found to inhibit preadipocyte-to-adipocyte differentiation in rats (Dieudonne et al., 2000). Castration increases differentiation in rat perirenal preadipocytes but inhibits differentiation of epididymal preadipocyctes, a process mediated through the IGF-1 receptor and PPARϒ (Lacasa et al., 1995). T and DHT have also been shown to inhibit adipogenic differentiation of 3T3-L1 and C3H10T1/2 cell lines (Singh et al., 2003, Singh et al., 2006). The adrenal androgen precursor DHEA inhibits murine 3T3-L1 preadipocyte proliferation, differentiation and ensuing lipid accumulation (Fujioka et al., 2012, Lea-Currie et al., 1998). We recently demonstrated that DHEA exerts a direct anti-glucocorticoid effect in the human preadipocyte cell line, Chub-S7, increasing basal glucose uptake and inhibiting preadipocyte proliferation and differentiation (McNelis et al., 2013).

In primary human tissue, Gupta *et al* found that DHT inhibits the differentiation of male human mesenchymal stem cells (hMSCs) into adipocytes, as well as differentiation of preadipocytes from three different fat depots (abdominal SC, mesenteric and OM) into mature adipocytes (Gupta et al., 2008). This effect was ameliorated by bicalutamide, an AR antagonist, in hMSCs. These findings were supported in recent work showing that T and DHT impair commitment of SC abdominal human adipose stem cells (hASCs) isolated from SC

adipose tissue of non-obese women to the preadipocyte phenotype, and also reduce preadipocyte differentiation to adipocytes (Chazenbalk et al., 2013). Mirroring this, Blouin *et al* found that both DHT and T markedly inhibited adipocyte differentiation in SC and OM primary human cultures in both sexes (Blouin et al., 2010).

It has been reported that distinct fat depots may differ in their differentiation response to androgens (Marin et al., 1996, Joyner et al., 2002, Dieudonne et al., 2000, Dieudonne et al., 1998). Increased androgen sensitivity is generally observed in visceral fat depots (Dieudonne et al., 1998, Lacasa et al., 1995). This phenomenon may be explained in part by the higher expression of AR in visceral fat compared to more superficial depots (Rodriguez-Cuenca et al., 2005, Joyner et al., 2002). Not all studies have been concordant with these observations, however; Blouin *et al* found no difference in androgen-induced inhibition of adipogenesis between SC and OM cultures in either sex (Blouin et al., 2010).

1.7.1.2 Androgens and lipid metabolism

Consistent with an inhibitory effect on adipogenesis, androgens have been shown to increase lipolysis. In rats, isoproterenol- and noradrenaline-stimulated lipolysis were increased by T (but not DHT) in male preadipocytes (Xu et al., 1990). In the same study, c-AMP-stimulated lipolysis was also stimulated by T. Catecholamine and cAMP-stimulated lipolysis in rats appear to be reduced by castration (Xu et al., 1991, Pecquery et al., 1988). The androgen precursor DHEA may also exert a key stimulatory effect on lipolysis in rats. Karbowska *et al* found that epididymal WAT from male rats treated with DHEA for 2 weeks had upregulation of ATGL and HSL mRNA and protein expression (Karbowska and Kochan, 2012). DHEA-treated animals also had higher serum FFA and glycerol levels compared to controls.

In humans, this effect may again be depot-specific. Basal lipolysis increased significantly *in vitro* in abdominal but not in gluteo-femoral adipose tissue obtained from female-to-male transsexuals receiving T treatment (Elbers et al., 1999). *In vivo*, catecholamine-stimulated lipolysis was increased by T in abdominal SC but not in femoral adipose tissue in men in another study (Rebuffe-Scrive et al., 1991). Dicker *et al* actually demonstrated inhibition of catecholamine-induced lipolysis by T in differentiated human SC (but not OM) preadipocytes, an effect possibly mediated by downregulation of β_2-adrenoreceptors (Dicker et al., 2004). Correlations have also been observed between plasma and OM fat tissue androgen levels, and OM adipocyte responsiveness to positive lipolytic stimuli (Belanger et al., 2006). Not all studies show concordant results in humans; in a recent study, androgens did not exert a stimulatory effect on either basal or stimulated lipolysis in paired OM and SC samples from men (Blouin et al., 2010). Gupta *et al* found that DHT exerted no effect on basal lipolysis in any depot, only significantly increasing forskolin-induced lipolysis in SC and mesenteric-derived preadipocytes, and not in those from the OM depot (Gupta et al., 2008). It is possible of course that this reduced lipolytic response of DHT-treated omental preadipocytes was the result of DHT-induced inhibition of adipogenic differentiation rather than a direct effect on lipolysis.

DHEAS, the inactive sulphated ester of DHEA, also exerted sex- and depot-specific effects on lipolysis in human WAT (Hernandez-Morante et al., 2008). DHEAS also increased glycerol release from visceral adipose tissue explants obtained from men, and in subcutaneous fat in women. These data, by inference, suggest the presence of the activating STS, which converts inactive DHEAS to active DHEA, in human adipose tissue, a supposition confirmed by Valle *et al* (Valle et al., 2006). However, a direct DHEA effect on adipose tissue is

unlikely, and these data may be reflective of intra-adipose conversion of DHEA to more potent downstream androgens.

Data on the effects of androgens on other processes integral to lipid accumulation, such as *de novo* lipogenesis, re-esterification or β-oxidation, are lacking, particularly in humans. In rats, treatment with cyproterone acetate had no significant effect on key esterification enzymes in subcutaneous, epididymal or mesenteric WAT (Hansson et al., 1991). In male chickens, castration increased expression of phosphoenolpyruvate carboxykinase (PEPCK), which regulates adipocyte and hepatic glyceroneogenesis (Duan et al., 2013). The paucity of data in this area merits future work.

1.7.1.3 Androgens and adipose insulin signalling

High T levels have been linked to cardiovascular risk factors and insulin resistance in women (Oh et al., 2002). It appears likely that some of the adverse metabolic effects of androgens are mediated by direct effects on adipose tissue insulin sensitivity (Ezeh et al., 2013). Chronic T treatment of female SC adipocytes impaired insulin-stimulated glucose uptake by impaired phosphorylation of protein kinase C (PKC) ξ. This effect was independent of PI3K and mediated via AR (Corbould, 2007). In non-human primates (NHPs), DHT reduced insulin-stimulated fatty acid uptake in female WAT, while *in vivo* T replacement potentiated insulin-induced Akt-phosphorylation in male WAT (Varlamov et al., 2012). This once again underpins key differences in regulation of insulin sensitivity by androgens in WAT of males and females. There is a distinct lack of extensive studies on the depot- and sex-specific effects of androgens on adipose tissue insulin sensitivity and molecular mechanisms remain to be elucidated.

Table 1-5 Androgen effects on adipocyte function: preadipocyte proliferation, adipocyte differentiation, lipolysis and insulin sensitivity.

	Androgen/ Treatment	**Model**	**Depot**	**Sex**	**Effect**	**Reference**
Preadipocyte proliferation	T, DHT	3T3-L1	N/A	N/A	↓	(Singh et al., 2003, Singh et al., 2006)
	DHEA	Chub-S7	N/A	N/A	↓	(Fujioka et al., 2012, Lea-Currie et al., 1998)
Adipocyte differentiation	T, DHT, DHEA	3T3-L1, C3H10T1/2, Chub-S7	N/A	N/A	↓	(Singh et al., 2003, Singh et al., 2006, McNelis et al., 2013)
	Castration	Rat	Epididymal	Both	↓	(Dieudonne et al., 2000)
	Castration	Rat	Perirenal	Both	↑	(Dieudonne et al., 2000)
	DHT	hMSCs	SC, OM, mesenteric	Male	↓	(Gupta et al., 2008)
	T, DHT	hASCs	SC	Female	↓	(Chazenbalk et al., 2013)
	T, DHT	Human preadipocytes	OM+SC	Both	↓	(Blouin et al., 2010)
Lipolysis	T, DHT	Rat preadipocytes	OM+SC	Male	↑ –	(Xu et al., 1991)
	DHEA	Rat WAT	Epididymal	Male	↑	(Karbowska and Kochan, 2012)
	T	Human SC *in vitro*	SC abdominal	Female-to-male transgender	↑	(Elbers et al., 1999)
	T	Human SC *in vivo*	SC abdominal	Male	↑	(Rebuffe-Scrive et al., 1991)
	T	Human preadipocytes	SC	Both	↓	(Dicker et al., 2004)
	T, DHT	Human preadipocytes	OM+SC	Male	–	(Xu et al., 1990)
	DHEAS	Human WAT	OM	Male	↑	(Hernandez-Morante et al., 2008)
	DHEAS	Human WAT	SC	Female	↑	(Hernandez-Morante et al., 2008)
Insulin sensitivity	T	Human adipocytes	SC abdominal	Female	↓	(Corbould, 2007)

1.7.1.4 Androgens and adipokines

There is abundant animal data to suggest that androgens may also modulate adipokine release by adipose tissue. Adiponectin is a 244-amino acid protein that is expressed exclusively in WAT (Halleux et al., 2001). It exists as three oligomeric complexes: high (HMW), middle (MMW) and low molecular weight (LMH) adiponectin (Xu et al., 2005). HMW adiponectin appears to be more important than the other oligomers in increasing insulin sensitivity and preventing atherosclerosis, and levels of this oligomer are lower in men than in women (Frias et al., 2001). Adiponectin is unusual among adipokines in that expression within adipocytes is downregulated in obesity and inversely correlated with body weight and adverse metabolic risk factors (Matsubara et al., 2002, Yang et al., 2001). Nishizawa *et al* demonstrated that T reduced total adiponectin secretion by 3T3-L1 adipocytes (Nishizawa et al., 2002). In the same study, a castration-induced increase in plasma adiponectin in male and female mice was associated with a significant improvement in insulin sensitivity. Both body fat and adiponectin are elevated in male andogen receptor knock-out (ARKO) mice compared to wild-type (Yanase et al., 2008), and after orchidectomy (Combs et al., 2003), which is an inverse pattern to the typical reduction in adiponectin that occurs with weight gain. Another study found that androgens selectively modulated the various oligomeric forms of adiponectin in mice (Xu et al., 2005); T reduced secretion of high molecular weight (HMW) adiponectin but not the low or middle molecular weight forms. The concentration of HMW adiponectin was significantly higher in females than in males. However Yarrow *et al* found that androgens reduce total circulating adiponectin concentrations in a dose-dependent manner, while maintaining HMW adiponectin, in both male and female rats (Yarrow et al., 2012).

In primary human cultures of abdominal SC and OM adipocytes, Blouin *et al* showed no significant inhibition of adiponectin secretion by androgens in male or female adipose tissue (Blouin et al., 2010). Interestingly, adiponectin concentrations are higher in hypogonadal men versus eugonadal men, despite the higher body fat associated with the former (Lanfranco et al., 2004).

1.7.1.5 Androgens and adipose metabolomics

There has been evolving interest in the last decade in the proteomic and metabolomic assessment of adipose tissue in health and disease. A recent study examined adipokine expression patterns in obese women with androgen excess compared to normo-androgenic controls and men (Martinez-Garcia et al., 2013). Women with androgen excess had adipokine expression profiles similar to those of men. Men and PCOS women had higher expression of chemerin and lipocalin-2 in OM compared to SC adipose tissue, with the opposite observation noted in control women. Putative androgen response elements (AREs) were found in the promoter regions of the genes encoding both adipokines. It is worth noting that chemerin and lipocalin-2 are key regulators in adipogenesis and adipocyte differentiation (Esteve et al., 2009). The same group further explored the potential ‘masculinization’ of female adipose tissue in the setting of androgen excess by comparing the adipose proteome of hyperandrogenic women to that of men and non-PCOS women (Montes-Nieto et al., 2013). Using a non-targeted proteomic approach, women with PCOS demonstrated masculinized patterns in the abundance of some proteins in OM and SC adipose tissue, including peroxiredoxin-6 (PRDX6) and creatine kinase B-type.

1.7.2 Pre-receptor androgen metabolism in adipose tissue

Adipose tissue is capable of active androgen synthesis and controls local availability by an intricate network of activating and inactivating enzymes (Figure 1-18) (Quinkler et al., 2004). The AKR1C enzyme subgroup of enzymes have emerged as important pre-receptor regulators of androgen metabolism in adipose tissue in recent years. We have already discussed the importance of the enzyme 5αR in peripheral steroidogenesis (Fassnacht et al., 2003), catalyzing the conversion of T to the highly potent androgen DHT. 5αR type 1 (*SRD5A1*) is expressed in adipose tissue (Wake et al., 2007) and may contribute to androgen production in fat. Adipose aromatization of androgens to oestrogen may also play a role in limiting local availability (Bulun and Simpson, 1994).

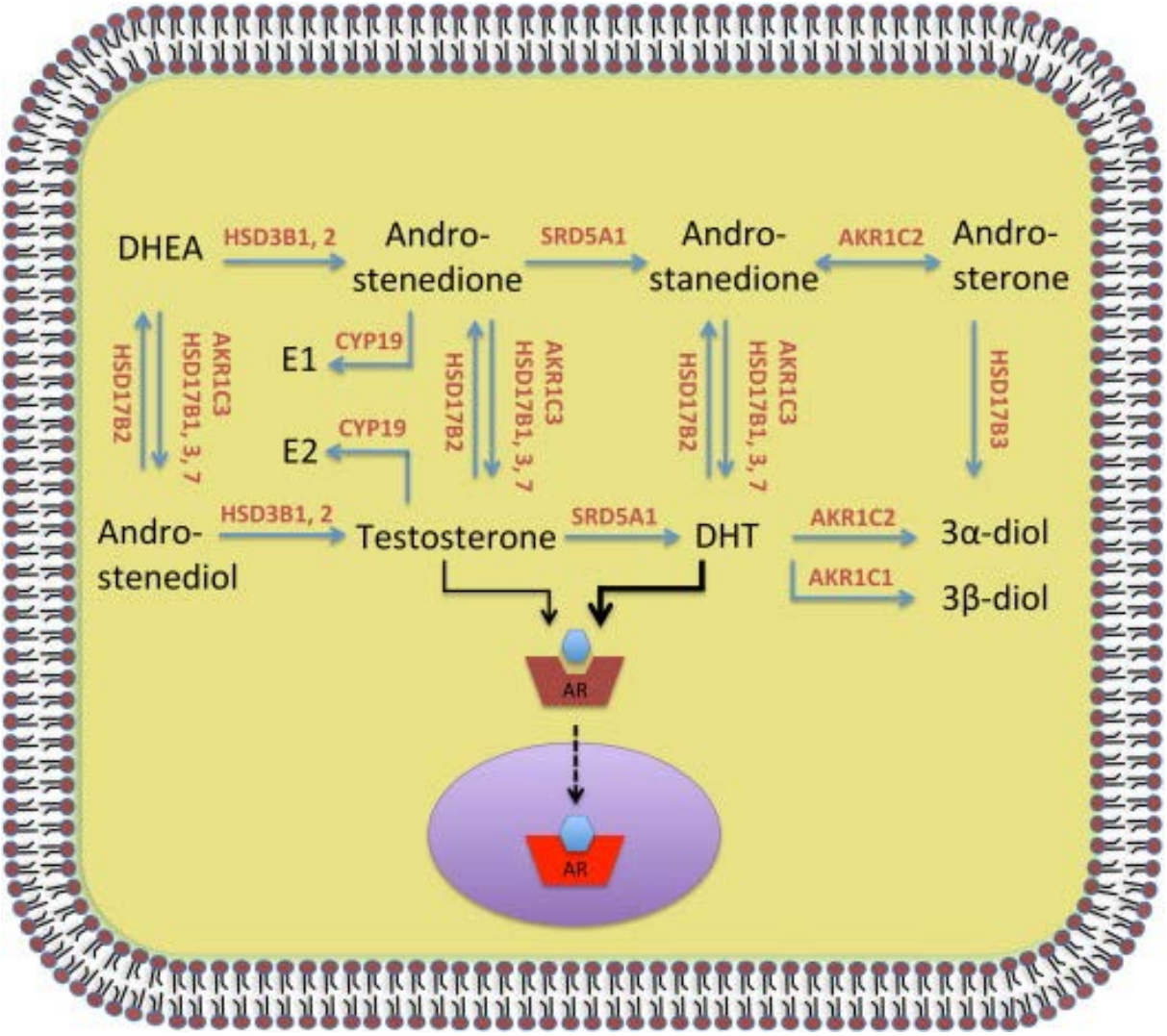

Figure 1-18 Pre-receptor androgen metabolism in adipose tissue (O'Reilly et al., 2014a). Active T is generated from the androgen precursor A by AKR1C3; DHT is generated from T by SRD5A1; AKR1C2 inactivates T to 5α-androstane-3α, 17β-diol. T and A are aromatized by CYP19A1 to E2 and E1 respectively. Reproduced with permission from Elsevier.

1.7.2.1 AKR1C isoenzymes

AKR1C isoenzymes are highly homologous but display different substrate specificities (Blouin et al., 2009b). AKR1C2 shares 86% of its amino acids with AKR1C3 and 98% with AKR1C1. AKR1C1, AKR1C2 and AKR1C3 display ketosteroid reductase

activities (20α-HSD, 3α-HSD, and 17β-HSD). AKR1C1 (also called 20α-HSD) predominantly inactivates progesterone into 20α-progesterone via 20α-HSD activity. It is expressed in both SC and OM adipose tissue in women but is not an important player in adipose androgen metabolism (Blanchette et al., 2005). AKR1C2 (also called 3α-HSD3) is predominantly involved in androgen inactivation, converting the potent androgen DHT into the weak 3α-diol through its 3α-reductase activity. Blouin *et al* described mRNA expression of AKR1C2 in human female adipose tissue (Blouin et al., 2003); they reported increased inactivation of 5α-DHT by AKR1C2 in omental fat from women with visceral obesity and suggested that local androgen inactivation is a predominant reaction in female abdominal tissue. Androgen-inactivating 3α-HSD activity has also been described in primary stromal cells and isolated adipocytes (Blouin et al., 2005, Blouin et al., 2006). Activity appears to be higher in subcutaneous compared to omental fat, and androgen inactivation correlated with obesity. Lower DHT levels in subcutaneous than in omental fat appears to support these findings (Belanger et al., 2006). Despite some discordant results between studies, it appears that subcutaneous fat is the key depot of androgen metabolism by AKR1C isoforms in males and females. All isoforms appear to be upregulated in obesity or with increased visceral adiposity (Quinkler et al., 2004, Blouin et al., 2005, Blouin et al., 2006). Wake *et al* also hypothesized that intra-adipose androgen metabolism by AKR1C isoforms drives central fat accumulation and may preferentially drive android compared to gynecoid fat distribution (Wake et al., 2007).

AKR1C3 (also called 17β-HSD5) is an androgen-activating isoform with predominant 17β-HSD activity, converting A into T (Zhang et al., 2000). AKR1C3 has emerged as a key mediator of androgen interconversion in the last 15 years (Penning et al., 2000, Dufort et al., 1999). Quinkler showed that AKR1C3 is upregulated in obesity, and that expression is

significantly higher in SC than in OM fat (Quinkler et al., 2004). AKR1C3 expression in SC fat reduced significantly in six women taking part in a weight-loss study. Other 17β-HSD isoforms capable of androgen generation have also been detected; Corbould *et al* detected low mRNA expression of isoenzymes 2 and 3 in human adipose tissue (Corbould et al., 1998), and subsequently found that preadipocytes readily converted A to T (Corbould et al., 2002).

1.7.2.2 Adipose tissue and 5αR activity

The most important isoenzyme of 5αR in terms of androgen activation and glucocorticoid inactivation is type 2 (*SRD5A2*), expressed predominantly in the epididymis, prostate and seminal vesicles; adipose tissue expresses only *SRD5A1* (Russell and Wilson, 1994). The precise biological role of 5αR1 is poorly understood. 5αR1 deletion in mice accelerates the development of hepatic steatosis but may protect against the development of NAFLD-related hepatocellular neoplasia (Dowman et al., 2013). This emphasizes its potential importance in lipid metabolism and accumulation. Whilst studies characterising the regulation and action of adipose 5αR1 are sparse, there is evidence that this enzyme plays an important role in fat. Levels of 5α-reduced metabolites are seen to increase with obesity in humans (Andrew et al., 1998), while weight-loss is associated with correspondingly decreased enzyme activity (Stimson et al., 2007, Tomlinson et al., 2008b). Increased activity of 5αR may act to reduce local levels of glucocorticoid by conversion to its tetrahydrometabolites and thus retain insulin sensitivity, but in females this beneficial effect may be offset by increased regional androgen activation.

There does appear to be an element sexual dimorphism in 5αR activity, with men showing greater activity as measured by 5α-reduced cortisol metabolites (Finken et al., 1999,

Tomlinson et al., 2008a); however levels of *SRD5A1* expression in SC adipose tissue were not found to differ between men and women (Tomlinson et al., 2008a). Differences in *SRD5A1* expression between OM and SC fat have not been demonstrated; however OM levels of DHT appear to be significantly higher, suggesting possible greater activity (Belanger et al., 2006).

1.7.2.3 Other androgen-metabolising enzymes in adipose tissue

Reports of adipose mRNA expression of a large number of androgen-metabolising enzymes have been published in recent years. 3β-HSD type 1, 17β-HSD types 2, 3 7 and 12, and 17α-hydroxylase are amongst potentially interesting isoforms expressed in fat (Kershaw and Flier, 2004, Corbould et al., 1998, Corbould et al., 2002, Puche et al., 2002, Bellemare et al., 2009), but detailed functional assessments and evaluation of clinical significance are for the most part lacking. The advent of LC-MS/MS offers the prospect of accurate adipose androgen quantification in health and disease, and early data are already exciting (Kinoshita et al., 2014).

1.8 Summary, hypotheses and aims

The role of adipose tissue as a potential source of androgen excess in PCOS has only been partially defined. The most recent body of work from key figures in the field suggests that SC and OM fat are host to an intricate network of androgen-activating and inactivating enzymes. The nature of the interaction between these enzymes in health and disease remains poorly understood. The ability of LC-MS/MS to sensitively and specifically elucidate androgen generation within fat is potentially exciting, but further work is needed to expand on embryonic developments. Current data on the impact of androgens on adipose lipid metabolism are conflicting and confusing, and focus largely on lipolysis. There are few published studies on the effects of androgens on other processes integral to lipid metabolism, such as beta-oxidation or free fatty acid uptake. The work undertaken in this doctoral thesis will attempt to clarify discrepancies in the literature and knowledge gaps in the area of adipose androgen metabolism using an integrated *in vivo*, *ex vivo* and *in vitro* approach. Specific hypotheses and study aims are listed below.

1.8.1 Adipose tissue is a key organ of androgen generation in PCOS

To test this hypothesis, I aim to:

- Examine mRNA expression of the androgen-activating enzyme AKR1C3 in primary human adipose tissue (Chapter 4). To control for the confounding effects of gender and depot-specificity, results will be compared between male and female patients, as well as between SC and OM fat depots.
- Explore the potential of the human preadipocyte cell line SGBS to activate the weak androgen precursor A to T. Results will be validated by replicating experiments in primary cultures of human adipocytes derived from SC and OM fat depots.

- Examine *in vivo* SC adipose androgen activation after oral administration of the androgen precursor DHEA in PCOS patients and healthy controls (Chapter 6).

1.8.2 Insulin is a key regulator of adipose and systemic androgen metabolism

The role of insulin in ovarian hyperandrogenism has been comprehensively studied in *in vivo* and *in vitro* models. However, evidence for regulation by insulin of systemic androgen metabolism is lacking. To test the above hypothesis, I aim to:

- Explore the association of the OGTT-derived insulin sensitivity index (ISI) with circulating androgen burden and urinary markers of systemic androgen metabolism (Chapter 3).
- Delineate the primary *in vivo* role of insulin in androgen generation by administration of oral DHEA to three patients with loss-of-function mutations of the INSR compared to PCOS women and controls (Chapter 6).
- Examine *in vitro* the role of insulin in adipose androgen generation by exposing primary adipocytes and SGBS cells to hyperinsulinaemic conditions, with measurement of AKR1C3 expression (Chapter 4). Functional effects of insulin on adipocyte androgen generation will be determined by LC-MS/MS.

1.8.3 Androgens play a crucial role in adipose tissue function, including adipogenesis and lipid metabolism

A role for androgens in adipose tissue function is suggested by sex-specific differences in body fat distribution. Studies on the role of androgens in adipocyte function

have yielded conflicting results, and are limited by sexual dimorphism and depot-specificity. In order to test the above hypothesis, I aim to:

- Perform a comprehensive *in vivo* study of the effects of androgens on female SC adipose tissue biology (Chapter 6).
- Examine effects of DHEA exposure on insulin-dependent processes in adipose tissue, such as lipolysis and glucose uptake (Chapter 6).
- Examine the role of androgens in adipose lipid metabolism and pancreatic insulin secretion *in vitro* (Chapter 5)

2 General Methods

The procedures described in this chapter are commonly used throughout this doctoral thesis. Any methods specific to individual chapters are described separately in the appropriate section. General chemicals were obtained from Sigma-Aldrich Company Ltd, Gillingham, Dorset, UK or Fisher Scientific, Loughborough, UK, unless otherwise stated. Reagents for reverse transcription and real-time polymerase chain reactions were obtained from Life Technologies Ltd, Paisley, UK, unless otherwise stated.

2.1 Primary human preadipocyte and adipocyte isolation

2.1.1 Principle

This method of adipocyte isolation from the stromo-vascular cell fraction using collagenase was first described by Rodbell in 1964 (Rodbell, 1964). The protocol described in this chapter is similar with only minor modifications. Incubation of adipose tissue with class 1 or 2 collagenase at 37°C breaks down connective tissue within fat, thereby releasing adipocytes and the stromo-vascular cell population. Subsequent centrifugation leads to separation of these two fractions into distinct layers, where fat-containing intact adipoyctes form a top layer and the stromo-vascular cells form a pellet at the bottom. Preadipocytes were cultured until confluent, at which point the process of differentiation was initiated by addition of chemically defined media (Figure 2-1).

2.1.2 Solutions

- Gibco® Dulbecco's Modified Eagle Medium:Nutrient Mixture (1:1) F-12 (DMEM/F-12, Life Technologies)
- Collagenase Type II from *Clostridium Histolyticum* (Sigma), 2mg/ml in DMEM/F-12
- Serum-free, chemically-defined medium: insulin (10nM), biotin 33µM, pantothenate 17µM and tri-iodothyronine (T3) 1nM. Medium was created according to well-established protocols in our laboratory (Bujalska et al., 1999).

2.1.3 Method

Paired primary human subcutaneous (SC) and omental (OM) fat samples were obtained from consented patients before elective abdominal surgery for non-malignant, non-inflammatory conditions. Sample collection was facilitated via the University of Birmingham Tissue Biorepository. Adipose tissue samples were dissected into 2-3mm^3 pieces and digested with type II collagenase at 37°C for 60 minutes. Samples were then centrifuged at 12,000 rpm for 5 minutes. Intact adipocytes were extracted using plastic Pasteur pipettes, and preadipocytes were extracted from a pellet containing the stromovascular components. Intact lipid-containing adipocytes were placed in serum-free DMEM/F12 and seeded into 1ml wells.

Preadipocytes were resuspended in DMEM/nutrient mixure F12 containing fetal bovine serum (FBS) 10% (Sigma Aldrich) and penicillin-streptomycin 1% (Life Technologies Ltd). Cells were seeded into 12- or 24-well plates depending on sample size (cells from 1g of adipose tissue per one 6-well plate, approximately 10,000 cells/cm^2) and incubated overnight. Cells were washed the next morning with serum-free DMEM/F12, after which they were again placed in serum-enriched growth media. Preadipocytes were allowed to proliferate to 90% confluence. Differentiation was initated by washing the cells in serum-free media, followed by culture with differentiation media containing biotin 33μM, pantothenate 17μM and T3 1nM. Cortisol (0.1μl/ml of 10^{-2}M stock), rosiglitazone (1μl/ml of 1mM stock) and insulin (1μl/ml of 1mg/ml stock) were added fresh (Bujalska et al., 2002). Methyl-3-isobutylxanthine (cyclic AMP-elevating agent, IBMX) was added fresh to the mixture for the first 4 days of differentiation only (5μl of 10mg/ml stock per ml of differentiation media). Chemically-defined media was changed at Days 4, 8 and 12.

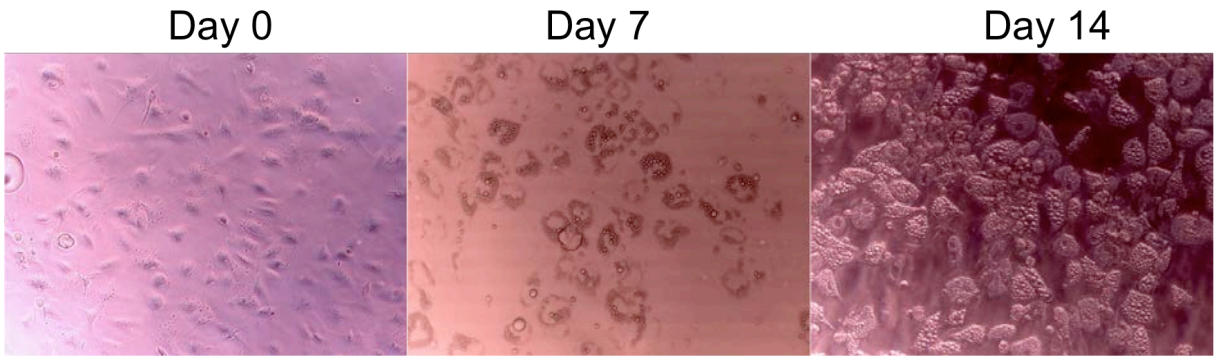

Figure 2-1 Preadipocyte differentiation primary fat.

2.2 Preadipocyte cell line

The human Simpson-Golabi-Behmel syndrome (SGBS) preadipocyte cell line was obtained from Professor Martin Wabitsch at the University of Ulm. Cells were differentiated into adipocytes according to previously published protocols (Fischer-Posovszky et al., 2008). The SGBS cell line was derived from the stromal cell fraction of SC adipose tissue from a male infant with Simpson-Golabi-Behmel syndrome (SGBS), a rare X-linked congenital overgrowth syndrome. We acknowledge the limitations associated with the use of a preadipocyte cell line in this doctoral thesis, including altered proliferation and differentiation capacity compared to primary human tissue. However, previous work has validated SGBS cells as a reasonable model for the study of human adipocyte biology.

2.2.1 Solutions

- 0F medium – DMEM/F12 mixture with 33µM biotin, 17µM pantothenate and penicillin-streptomycin 1%.
- Growth medium – 0F medium supplemented with FBS 10%

- Quick Diff medium – 0F medium with transferrin (0.01mg/ml), insulin (20nM), cortisol (100nM), T3 (0.2nM), dexamethasone (25nM), IBMX (250µM) and rosiglitazone (2µM) (all reagents Sigma Aldrich Company Ltd)
- 3FC medium – 0F medium with transferrin (0.01mg/ml), insulin (20nM), cortisol (100nM) and T3 (0.2nM)

SGBS cells were grown to 90% confluence in 5ml flasks. Trypsin-EDTA (0.25%, Life Technologies) 1ml was added for dissociation. Cells were suspended in growth medium, centrifuged for 10 minutes at 12,000 rpm and then resuspended. SGBS preadipocytes were seeded into 12- or 24-well plates at a density of 100,000/cm^2. At 90% confluence per well, growth media was removed and cells washed three times with 0F media. Quick Diff media was added for 4 days; at Day 4, media was changed to 3FC until Day 14 (changed at day 4, day 8 and day 12).

2.3 MIN6 mouse insulinoma cell line

An established mouse pancreatic β-cell line was obtained as a gift from Professor Kevin Docherty at the University of Aberdeen, UK (Miyazaki et al., 1990). Cells were cultured in Dulbecco's modified Eagles' Medium (DMEM) with glucose 4.5g/l, 10% FBS (Sigma Aldrich), 1% penicillin/streptomycin (Sigma Aldrich) and 100µM beta-mercaptoethanol. Cells were incubated at 37°C.

2.4 Realtime quantitative PCR

2.4.1 RNA extraction and quantification

Total RNA was extracted from cells after treatments as appropriate using the Tri-Reagent system (Sigma). All steps were performed on ice to preserve RNA integrity. Typically, 1ml of Tri-Reagent was added to wells per 10^7 cells as recommended by manufacturer. Cells were then incubated at room temperature for 10 minutes to allow cells to homogenise completely. RNAse-free chloroform was added to samples, which were then mixed vigorously and incubated at room temperature for 10 minutes before centrifugation at 13,000 rpm for 20 minutes at 4°C. The upper aqueous phase containing RNA was transferred to a fresh 1.5ml tube. RNA was precipitated by incubating samples in 500µl isopropranolol for 16 hours at -20°C. The resultant RNA pellet was washed in 70% ethanol twice, followed by centrifugation, and resuspended in 20-30µl of RNase-free water. RNA concentration and purity was calculated using NanoDrop technology (ND1000, ThermoScientific, Wilmington, DE, USA). Total RNA was extracted from whole adipose tissue explants by homogenizing 100-200mg of adipose tissue in Tri-Reagent using an Ultra Turrax homogenizer (Cole-Palmer, UK). Further steps were carried out as outlined above.

2.4.2 Complementary DNA synthesis and amplification – reverse transcription

Reverse transcription is the process of converting single stranded RNA into complementary DNA (cDNA). RNA was diluted to a final concentration of 50-100ng/µl. Reverse transcription was carried out using Taqman Reverse Transcription Reagents (Applied Biosystems/Life Technologies Ltd). In this protocol, gRNA was reverse transcribed into cDNA using the following reagents:

- 10XRT buffer
- 25mM $MgCl^2$
- dNTP mix (10mM each)
- RNase inhibitors
- Multiscribe Reverse Transcriptase (50U/µl)

Total RNA is heated to denature secondary structures, followed by cooling which facilitates annealing of random hexamers. After annealing, and in the presence of an RNase inhibitor, these are extended with reverse transcriptase. Single stranded cDNA can then be used in a PCR reaction. All reactions were run according to the following cycle programme:

- 25°C for 10 minutes
- 37°C for 60 minutes
- 48°C for 30 minutes
- 95°C for 5 minutes

2.4.3 Realtime PCR

2.4.3.1 Principle

Taqman Realtime PCR technology employs a fluorogenic probe which allows detection of a specific PCR product as it accumulates in 'real time' during the PCR reaction. An oligonucleotide probe is synthesized containing fluorescent reporter dye on its 5' end and a quencher dye on its 3' end. The quencher dye reduces fluorescence emitted by the report

dye. The probe anneals downstream from one of the primer sites and during primer extension is cleaved by the 5' nuclease activity of Taq DNA polymerase. Removal of the probe facilitates primer extension, separating the reporter and quencher dye, thereby allowing the reporter dye signal to increase. Additional reporter dye molecules are cleaved during successive cycles, which allows the fluorescence intensity to increase proportional to the amount of PCR product generated. The method detects when the target is first detected rather than the amount of target produced after a fixed number of cycles. The value at which the product is first detected is referred to as the Ct value. The higher the target copy number in the sample, the lower the cycle number when fluorescence is first observed, therefore the lower the Ct value. This allows relative quantification compared to a housekeeping gene (see below).

2.4.3.2 Method

mRNA expression levels were determined using an ABI sequence detection system (Perkin-Elmer Applied Biosystems, Warrington, UK). All reactions were carried out in 10µl final volume in reaction buffer, containing 2 X Taqman Universal PCR Master mix (5.0µl, Applied Biosystems), probe-primer mix for gene of interest (0.5 µl) and 4.5µl cDNA (100ng) (Gathercole et al., 2007). All reactions were normalized against the housekeeping genes 18S rRNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data were expressed as the cycle number at which logarithmic PCR plots crossed a calculated threshold line (Ct values) and used to determine Δct values [Δct=(ct of target gene)-(ct of housekeeping gene)]. Arbitrary units were calculated using the formula ($1000*2^{-\Delta ct}$). All statistics were performed on Δct values.

2.5 Steroid extraction and measurement by liquid chromatography/tandem mass spectrometry (LC-MS/MS)

2.5.1 Serum steroid extraction and measurement

Serum steroids were measured by liquid chromatography/tandem mass spectrometry (LC-MS/MS) using a Waters Xevo mass spectrometer with Acquity uPLC system. LC-MS/MS conditions were an electrospray ionization source with capillary voltage 4.0kV, a source temperature of 150°C, and a desolvation temperature of 500°C. A linear calibration series of increasing concentrations was used, and internal standard solution added to both samples and calibrants. Serum steroid oxime analysis was employed using the technique described by Kushnir (Kushnir et al., 2010). This technique facilitates enhanced detection of androgens at low serum concentrations by formation of oxime derivatives of the steroid oxo-groups. Measurement of T, A and DHEA was carried out in positive mode, whereas measurement of DHEAS was performed in negative mode. Steroids were extracted from 200µl serum via liquid-liquid extraction using 1ml tert-butyl-methyl-ether (MTBE), with subsequent derivitisation into steroid oximes using 100µl derivitisation cocktail (0.16g hydroxylamine in 8ml pyridine). DHEAS was extracted by adding 20µl $ZnS0_4$ 0.1mM and 100µl acetonitrile to 20µl serum for protein precipitation, and subsequent evaporation under constant nitrogen flow (Chadwick et al., 2005). All steroids were separated using an optimized gradient system consisting of methanol with 0.1% formic acid. Quantification was carried out by reference to a linear calibration series with appropriate internal standards. Individual steroids were identified by matching retention times and two mass transitions in comparison to a deuterated reference compound.

2.5.2 Cell media steroid extraction and measurement

After treatment with androgens or androgen precursor, cell media (1-2ml) was extracted from tissue culture wells and placed in sylinised glass TLC tubes. For quantitative analysis, 20µl internal standard was added. A calibration series was prepared for each experiment as follows:

Table 2-1 Sample calibration series for cell media steroid measurement

Calibration number	**Added volume of serum steroid stock 2 (µl)**	**Added volume of cell media (µl)**	**Concentration (ng/ml)**
C0	0	1000	0
C1	0.5	999.5	0.5
C2	1	999	1
C3	5	995	5
C4	10	990	10
C5	25	975	25
C6	50	950	50
C7	100	900	100
C8	250	750	250
C9	500	500	500

Steroids were extracted via liquid-liquid extraction as described above by adding 3ml MTBE to each sample. After vortexing and centrifugation at 12,000rpm for 5 minutes, samples were frozen at -20°C for least 60 minutes. Subsequently, the upper clear layer was transferred to a 96-well plate using glass Pasteur pipettes, with alternating cycles of

evaporation at 55°C under nitrogen followed by further liquid transfer until the samples were evaporated in entirety. After evaporation, samples were reconstituted with 125µl of methanol:H_20 (50:50) and frozen at -20°C until ready for LC-MS/MS analsysis.

2.5.3 Urinary steroid metabolite excretion analysis

Urinary steroid metabolite excretion from 24-hour urine samples was analysed by experienced laboratory staff with gas chromatography/mass spectrometry (GC/MS) using the techniques described by Shackleton and colleagues (Arlt et al., 2004, Shackleton, 1993). Steroids were released from conjugation enzymatically before extraction; individual steroid metabolites were identified by Selected-Ion-Monitoring analysis.

First, steroids were extracted from urine with a SepPak column (Waters, Milford, Ma, USA): 5 ml of urine sample were loaded after washing the columns with 4 ml methanol and 4 ml of ddH_2O. Then, the loaded column was washed with 4 ml ddH_2O and the steroids were eluded in 4 ml methanol into a clean borosilicate tube. After evaporation (heat block at 55^{o}C under N_2), sulfate and glucoronide groups were enzymatically removed from conjugated steroids by incubating for 3 hours at 55^{o}C in the hydrolysis buffer (3 ml 0.1M acetate buffer [pH 4.8-5.0] + 10 mg ascorbate + 10 mg sulfatase/glucoronidase; all ingredients purchased from Sigma, Pool, UK). The cooled sample was then loaded again on a SepPak column and eluded into a clean glass tubes with 4 ml methanol. Derivatization: After evaporation, three drops of 2% methoxyamine-pyridine was added; the tube was vortexed vigorously and incubated at 55^{o}C for one hour. After evaporation under N_2, 75µl of N-trimethylsilylimidazole (Sigma, Pool, UK) were added, vortexed and incubated at 120^{o}C overnight. Then, the samples were extracted by adding (1) 2 ml cyclohexane and (2) 2 ml

dH_2O (vortexing after each step). After centrifugation (1,000g for 5 minutes), the bottom later layer was removed into waste. Then, another 2 ml dH_2O were added, vortexed, centrifuged and removed. Finally, the top layer containing the extracted in cyclohexane was transferred into injection vials. The samples were then injected into an Agilent 5973 GC mass-spectrometer.

The sum of androsterone (An) and etiocholanolone (Et) was considered representative of active androgen metabolite excretion (Silfen et al., 2002). Total body 5α-reductase activity was estimated using the ratios of An/Et and 5α-THF/THF. Activity of systemic 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) was calculated using the ratios of tetrahydrometabolites of cortisol (5α-THF+THF) to tetrahydrocortisone (THE) (Stewart et al., 1999), while 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) activity was estimated by the ratio of urinary free cortisol to cortisone (UFF/UFE). Total glucocorticoid metabolite excretion was assessed as the sum of 5α-THF + THF + THE + cortolones + cortols + cortisol + cortisone.

2.6 Serum insulin, glucose and free fatty acid measurements

2.6.1 Human and Mouse insulin ELISA kit (Mercodia, Sweden)

This is a solid phase two-site enzyme immunoassay using two monoclonal antibodies (abs) against separate antigenic determinants on the insulin molecule. Each kit contains the following:

- Mouse monoclonal anti-insulin-coated plate
- Calibrators 0-5 containing set concentrations of recombinant human insulin
- Enzyme Conjugate 11X (1.2ml) –mouse monoclonal anti-insulin

- Enzyme Conjugate Buffer (12ml) – added to Enzyme Conjugate 11X to make Enzyme Conjugate 1X solution
- Wash buffer 21X (50ml) – added to 1L distilled H_20 to make wash buffer 1X solution
- Substrate TMB (light sensitive)
- Stop solution

Human serum or cell media from mouse MIN6 cells was defrosted on ice at room temperature. Calibrators and samples (10µl each) in duplicate were transferred to 96-well plate. Enzyme conjugate 1X solution 100µl was added to each well, and samples incubated on a plate shaker (700-900 rpm) for 2 hours at room temperature (18-25°C). Reaction volume was discarded by inverting the microplate over a sink and plates then washed manually by adding 350µl wash buffer 1X solution to each well. Wash solution was discarded and the plate tapped firmly against absorbent paper to remove excess liquid. This step was repeated 5 times, followed by addition of Substrate TMB 200µl to each well. After incubation for 15 minutes at room temperature, 50µl Stop Solution was added and the plate placed on the shaker for 5 seconds to ensure mixing. Optical density of plates was measured at 450nm on spectrophotometer.

2.6.2 Serum free fatty acids

Serum free fatty acids were measured in human serum samples using a commercially available kit (ZenBio, NC, US). Assessment of serum fatty acids is via a coupled reaction to measure non-esterified fatty acids (NEFA). The initial step is catalyzed by acyl-CoA synthetase (ACS), which produces fatty acyl-CoA thiol esters from the NEFA, ATP, magnesium and CoA in the reaction. Subsequently acyl-CoA esters react with oxygen in the presence of acyl-CoA oxidase to produce hydrogen peroxide. In the presence of peroxidase,

hydrogen peroxide allows the oxidative consensation of 3-methyl-N-ethyl-N-(β-hydroxylethyl)-aniline with 4-aminoantipyrine, forming a purple product which absorbs light at 550nm.

2.6.2.1 Solutions

- Dilution buffer 100ml
- Free fatty acid (FFA) standard
- FFA Diluent A
- FFA Diluent B
- FFA Reagent A
- FFA Reagent B

2.6.2.2 Procedure

1. A standard curve was prepared using the Standard Solution by means of serial dilutions with dilution buffer, with final concentrations of 0, 1.4, 4.1, 12.3, 37, 111 and 333µM.

2. FFA Reagent A and B was prepared by adding 50ml FFA Diluent A and B respectively.

5µl of serum samples were added in duplicate to a 96-well plate. 45µl of dilution buffer was added to each well to give a total volume of 50µl (1:10 dilution).

3. 100 µl of Reagent A was added to each well. The plate was placed on an orbital shaker for 10 seconds to ensure mixing, then placed in an incubator at 37°C for 10 minutes.

4. 50 µl of Reagent B was next added to each well and the plate placed on an orbital shaker for 10 seconds to ensure mixing. The plate was then placed in an incubator at 37°C for 10 minutes

5. The plate was allowed to equilibrate at room temperature for 5 minutes.

6. The optical density of each well was then measured at 540nm.

2.6.3 Measuring glucose using near-patient YSI machine

Serum glucose was measured using the 2300 STAT PLUS Analyser (YSI Incorporated, Life Sciences, Ohio, USA).

Required equipment:

- 2 ml plastic tubes to present to the sipper
- Rinsing buffer
- Calibrators

After calibration, the YSI machine was placed in 'RUN' mode. Serum samples were automatically aspirated and glucose result displayed on screen in 15-30 seconds. The YSI automatically self-calibrates every 15 minutes when in RUN mode.

2.7 Body composition assessment using DXA

Dual-energy x-ray absorptiometry (DXA) was employed for body composition assessment using Hologic Discovery/W DXA (software version Apex 3.0, Hologic Inc.). Specific fat phenotype was measured using android, gynecoid, peripheral (arms and legs), and trunk regions of interest, with subsequent calculation of android:gynecoid and trunk:peripheral fat ratios (Gregson et al., 2013).

2.8 Statistical analysis

Experimental data were graphically presented and statistically analysed with Prism 6.0 (CA, USA), SPSS Statistics (Version 22.0, IBM Corp., Armonk, NY, USA) and Microsoft Excel for Mac 2011 (Version 14.4.5). Independent samples *t* tests were used to compare means of two different groups when samples were drawn from normally distributed populations with the equal variance. Mann-Whitney Rank Sum test was used when two groups were not drawn from a normally distributed population. P-values, meaning the probability of having the observed data if the null hypothesis is true (ie difference occurring by chance), were calculated using the above tests; $P<0.05$ was deemed to be statistically significant.

When comparing means from multiple groups, one-way ANOVA with post-hoc Tukey testing was employed for multiple comparisons between different groups. Correlation testing was employed to examine positive and negative linear relationships between continuous variables, using Pearson's test (parametric) or Spearman's rank test (non-parametric data) as appropriate. Multiple linear regression was used to examine relationships between continuous variables while adjusting to the effects of confounding factors.

3 Circulating androgens and metabolic phenotype in PCOS

Parts of this chapter have been published as:

Hyperandrogenemia predicts metabolic phenotype in polycystic ovary syndrome: the utility of serum androstenedione

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3.1 Introduction

A lack of clarity persists on which androgens should be measured in PCOS, what constitutes normal reference ranges and which analytical technique should be employed (Barth et al., 2007). Equally we have little insight into whether different patterns of biochemical hyperandrogenemia define distinct PCOS sub-groups in terms of metabolic risk. The most accurate strategy for quantification of biochemical androgen excess in PCOS is measurement of total 24-h urinary androgen metabolite excretion (Silfen et al., 2002); however, urinary steroid profiling by gas chromatography/mass spectrometry (GC/MS) is time-consuming and expensive. In routine clinical practice, and as defined by PCOS diagnostic criteria, serum total T remains the most commonly measured and widely available marker for estimation of biochemical androgen excess (Azziz et al., 2004). However, measurement of T is severely limited by a number of factors: (i) T circulates bound to SHBG and other proteins such as albumin, and only the unbound or free fraction enters into target tissues (Pardridge, 1986), such that circulating levels may not represent tissue exposure, (ii) measurement of free T by direct RIA is highly inaccurate (Vermeulen et al., 1999), resulting in the recommendation to calculate the free androgen index from T and SHBG instead, (iii) direct immunoassays for total T are highly variable (Boots et al., 1998), and (iv) T circulates in low nanomolar range concentrations and the lower end of the normal female reference range is below the limit of quantitation in all assays available, including tandem mass spectrometry (LC-MS/MS).

Androgen excess in PCOS originates from ovaries and adrenals (Loughlin et al., 1986); moreover, increased peripheral conversion of T to the most potent androgen 5α-DHT by systemically upregulated 5αR activity has been well-documented (Stewart et al., 1990, Fassnacht et al., 2003). A is the immediate precursor of T. In addition to direct secretion by

ovaries and adrenals, A can be generated in peripheral tissues from its precursor DHEA by the enzyme 3β-HSD2 (Ehrmann et al., 1995). The vast majority (95-97%) of DHEA and its sulfated ester DHEAS are secreted by the adrenocortical zona reticularis and elevated serum levels are observed in 20-30% of women with PCOS (Kumar et al., 2005). In addition to mainly ovarian production, about 25% of plasma testosterone derives from the conversion from A by 17β-HSD5 (also called AKR1C3) in adipose and other peripheral tissues (Quinkler et al., 2004). Serum A is inconsistently measured in routine clinical investigation of suspected PCOS as clinicians remain uncertain about its additional diagnostic value or clinical utility. Limited previous work suggests that 10% of PCOS patients may be misclassified as normoandrogenemic if A is not measured (Knochenhauer et al., 1998).

The independent health risks of androgen excess in women are increasingly apparent (Jones et al., 2012). We hypothesized that patients with co-elevation of A and T and therefore increased circulating androgen burden represent a distinct subgroup with adverse metabolic parameters compared to those with normal androgens or solitary elevation of either A or T. In women, A circulates at ten-fold higher concentrations than serum T (Horton et al., 1966) and is therefore more easily and accurately detected than T, not only by mass spectrometry but also by widely-available immunoassays. We thus also hypothesized that A represents a more sensitive marker for the biochemical detection of androgen excess. We therefore carried out a detailed analysis of androgen synthesis and metabolism to test these hypotheses in a large cohort of women with PCOS according to Rotterdam criteria in comparison to a group of BMI-matched healthy controls.

3.2 Materials and methods

3.2.1 Research strategy

In this chapter, we will examine the metabolic phenotype of a large cohort of PCOS and control patients according to their circulating androgen burden as measured by LC-MS/MS. Insulin sensitivity indices, as determined by OGTT, and other metabolic markers such as lipids and blood pressure, will be correlated with serum androgens and 24-hour urinary steroid excretion. The relationships between central and peripheral fat mass distribution with markers of androgen metabolism and insulin sensitivity will also be explored.

We will test the hypotheses that (1) women with co-elevation of T and the androgen precursor A have a higher metabolic risk than those with solitary androgen derangements, and (2) that A is a more sensitive biochemical marker of androgen excess in PCOS than conventional T. Lastly, we will examine the utility of application of the current Rotterdam classification criteria as a tool to predict the prevalence of insulin resistance and dysglycaemia in PCOS.

3.2.2 Subjects

Women with PCOS were recruited from outpatient clinics at the Queen Elizabeth Hospital Birmingham (QEHB) and Birmingham Women's Hospital (BWH). This observational, cross-sectional study was approved by South Birmingham Research Ethics Committee (REC) and all participants gave written informed consent. PCOS was diagnosed according to the Rotterdam ESHRE 2004 criteria, with the presence of two or more of the following: oligo-/anovulation, clinical signs of hyperandrogenism, and polycystic ovaries on

ultrasound (Rotterdam, 2004b). Other causes of oligomenorrhea and/or androgen excess were excluded by history, physical examination, biochemical assessment and, where appropriate, imaging. Healthy controls were recruited via local advertisement, with a diagnosis of PCOS excluded by clinical and biochemical parameters. Exclusion criteria for both groups were current or recent treatment with glucocorticoids, CAH, hyperprolactinemia, thyroid dysfunction, pregnancy, age <18 or >45 years, oral contraceptive use within 3 months prior to recruitment, and diabetes mellitus.

3.2.3 Clinical protocol

Participants attended the National Institute of Health Research/Wellcome Trust Clinical Research Facility at the Queen Elizabeth Hospital Birmingham after an overnight fast for a single day of integrated assessment. Each patient provided a 24-h urine sample for urinary steroid metabolite analysis by gas chromatography/mass spectrometry (GC/MS) as previously reported (Arlt et al., 2004). Baseline anthropometric assessment included height, weight, body mass index (BMI, kg/m^2), waist:hip ratio and blood pressure (BP) measurement. A modified Ferriman-Gallwey scoring system for hirsutism was used, where each of nine body areas were rated from 0 (absence of terminal hairs) to 4 (extensive terminal hairs) (Yildiz et al., 2010). Dual-energy x-ray absorptiometry (DXA) was employed for body composition assessment using Hologic Discovery/W DXA (software version Apex 3.0; Hologic Inc., Bedford, MA). Specific fat phenotype was measured using android, gynaecoid, peripheral (arms and legs) and trunk regions of interest, with subsequent calculation of android:gynaecoid and trunk:peripheral fat ratios (Gregson et al., 2013).

Bloods were drawn for fasting plasma glucose and insulin and serum T, A, DHEA, DHEAS, SHBG, FSH and LH, total cholesterol (TChol), high-density lipoprotein (HDL) and triglycerides (TG). Each participant underwent a 75-g OGTT with blood sampling at 30-minute intervals for 2 hours. Glucose and insulin were measured using methods described previously (Vassiliadi et al., 2009) and in secton 2.6. Subjects were categorized as having normal glucose tolerance (NGT) or dysglycemia on the basis of 2-h glucose values (NGT <7.8mmol/L, dysglycemia ≥7.8mmol/L). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula [fasting glucose (mmol/L)*fasting insulin (mIU/L)/22.5]. HOMA-IS (insulin sensitivity) was calculated using the formula 100/HOMA-IR. Insulin sensitivity index (ISI) was calculated using the formula [75,000 + ($Gluc_0$-$Gluc_{120}$)*1.15*180*0.19*body weight (kg)]/120*log(Ins_{mean})*$Gluc_{mean}$] (mg*L^2*/mmol*mU*min) (Cederholm and Wibell, 1990, Stumvoll et al., 2000).

3.2.4 Serum and urine steroid measurements

Serum steroids were measured by liquid chromatography/tandem mass spectrometry (LC-MS/MS) employing a Waters Xevo mass spectrometer with Acquity uPLC system, as described in section 2.5.1. Briefly, LC-MS/MS conditions were an electrospray ionization source with capillary voltage 4.0kV, a source temperature of 150°C, and a desolvation temperature of 500°C. Serum steroid oxime analysis, which facilitates enhanced detection by formation of oxime derivatives of the steroid oxo-groups (Kushnir et al., 2010), was employed for the measurement of T, A and DHEA and carried out in positive mode, whereas measurement of serum DHEAS was performed in negative mode. T, A and DHEA were extracted from 200μl serum via liquid-liquid extraction using 1ml tert-butyl-methyl-ether

(MTBE) followed by derivatization into steroid oximes employing 100µl derivatization mixture (0.16g hydroxylamine in 8ml pyridine). For protein precipitation and extraction of DHEAS, 20µl $ZnSO_4$ 0.1mM and 100µl acetonitrile were added to 20µl serum before evaporation under constant nitrogen flow (Chadwick et al., 2005). All steroids were separated using an optimized gradient system consisting of methanol with 0.1% formic acid and quantified referring to a linear calibration series with appropriate internal standards. Each steroid was identified by matching retention times and two mass transitions in comparison to a deuterated reference compound.

Serum A and T were categorized as normal based on locally-derived LC-MS/MS reference values for premenopausal women (T, 0.3-1.9nmol/L; A, 1.4-7.4nmol/L; 2.5^{th}-97.5^{th} percentiles of an unselected healthy female control cohort). Serum androgens were used to categorize PCOS patients into four subgroups: normal A/normal T (NA/NT), high A/normal T (HA/NT), normal A/high T (NA/HT) and high A/high T (HA/HT). The free androgen index (FAI) was calculated in each patient using the formula (T*100)/SHBG. In an additional analysis, we also subdivided the PCOS patients into the four phenotypic subgroups arising from the Rotterdam criteria recommended at a recent NIH meeting (Fauser et al., 2012). These subgroups are defined by the presence of (i) androgen excess, anovulation and polcystic ovaries on ultrasound (AE+Anov+PCO), (ii) Anov+PCO, (iii) AE+PCO and (iv) AE+Anov.

Urinary steroid metabolite excretion analysis was carried out by GC/MS as described previously (Arlt et al., 2004) and as summarized in section 2.5.3. The sum of androsterone (An) and etiocholanolone (Et) was considered representative of active androgen metabolite excretion (Silfen et al., 2002). Net systemic 5α-reductase activity was assessed by the ratios of An/Et and 5α-tetrahydrocortisol/tetrahydrocortisol (5α-THF/THF). 11β-hydroxysteroid

dehydrogenase type 1 (11β-HSD1) activity was estimated using the ratio of the tetrahydrometabolites of cortisol (5α-THF + THF) to tetrahydrocortisone (THE) (Stewart et al., 1999) and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) activity by the ratio of free urinary cortisol to cortisone (UFF/UFE). Total glucocorticoid metabolite excretion was assessed as the sum of 5α-THF + THF + THE + cortolones + cortols + cortisol + cortisone.

3.2.5 Statistical analysis

Statistical Package for the Social Sciences (SPSS, Chicago, Il.) Version 21 was used for data analysis. All data is expressed as mean ± standard deviation (SD) unless otherwise stated. Data was log-transformed if necessary to normalize before using parametric tests. Independent samples *t* tests or Mann-Whitney were used as appropriate for comparison between two groups. One-way ANOVA with *post hoc* Tukey testing was used for multiple comparisons between different groups. Categorical variables were compared using Pearson's Chi-Square test. Correlation testing was performed using Pearson's correlation co-efficient for normally distributed data or Spearman's test for non-normally distributed data. Multiple linear regression was used to adjust for the confounding effects of different variables. Differences were considered statistically significant at $p<0.05$.

3.3 Results

Baseline clinical and metabolic characteristics of PCOS and age- and BMI-matched healthy controls are shown in Table 3-1. Complete clinical, biochemical and radiological data were available in 86 women with PCOS, all fulfilling the clinical phenotype criteria for PCOS as defined by the Rotterdam consensus (Rotterdam, 2004b). Results were compared to those obtained from 43 age- and BMI-matched controls. Hirsutism and oligomenorrhea were present in 87% and 92% of PCOS patients, respectively. Polycystic ovaries were present on ultrasound in 73%. PCO subgroups arising from the Rotterdam criteria were also matched for age and BMI and were categorized as per the following clinical phenotype groups: (i) AE, Anov, PCO (n=51, 59%), (ii) AE+PCO (n=6, 7%), (iii) Anov+PCO (n=9, 11%) and (iv) AE+Anov (n=20, 23%).

Table 3-1 Clinical, biochemical and metabolic characteristics in the overall PCOS cohort (n=86), PCOS androgen phenotype subgroups and age- and BMI-matched controls (n=15). PCOS subgroup classification according to high (H) or normal (N) serum androstenedione (A) and serum testosterone (T) concentrations as measured by liquid chromatography/tandem mass spectrometry (normal T (NT) ≤1.9nmol/l; normal A (NA) ≤7.4nmol/l). Normal glucose tolerance, 2-h glucose oGTT ≤7.7mmol/l. Dysglycemia, 2-h glucose oGTT ≥7.8mmol/l.

a, p<0.05, b, p<0.01, c, p<0.001 as compared to BMI-matched healthy controlsd. d, p<0.05; e, p<0.01; f, <0.001 as compared to NA/NT group

Variable	**Controls (n=43)**	**All PCOS (n=86)**	**NA/NT (n=10)**	**HA/NT (n=20)**	**HA/HT (n=56)**
Age (years)	32.4±9.8	30.0±7.2	32.9±2.9	30.8±7.9	28.8±7.0
BMI (kg/m^2)	30.3±6.4	31.9±7.1	30.2±6.5	30.0±5.9	32.1±6.5
Ferriman-Gallwey Score	2.1±1.6	12.4±6.7[c]	9.9±5.3[c]	10.1±6.4[c]	13.9±7.2[c,d]
Testosterone (nmol/L)	1.3±0.4	2.2±0.8[c]	1.2±0.3	1.6±0.4	2.6±0.7[c,f]
FAI (Tx100/SHBG)	3.2±1.8	9.1±6.1[c]	5.5±3.9[a]	7.3±4.6[a]	10.5±6.5[c,d]
SHBG (nmol/L)	49.8±26.6	32.3±18.4[c]	28.8±11.8[c]	30.8±21.2[c]	31.8±16.5[c]
Androstene-dione (nmol/L)	3.5±1.4	13.4±5.6[c]	5.1±1.7	11.4±4.2[c,f]	14.2±5.1[c,f]
DHEA (nmol/L)	20.5±7.9	57.5±30.0[c]	24.4±8.6	59.8±34.8[c,e]	58.0±26.8[c,e]
DHEAS (µmol/L)	3.9±2.0	6.2±3.0[c]	2.4±1.2	6.3±2.6[f]	6.2±2.7[c,f]
DHEA/ DHEAS ratio	7.8±3.7	12.9±6.8[a]	12.6±8.5[a]	14.0±10.0[a]	15.4±5.4[a]
LH (IU/L)	5.2±2.4	9.9±5.8[a]	4.7±3.2	9.0±6.3[a]	11.4±9.2[a,d]
LH/FSH ratio	0.8±0.5	2.0±1.5[a]	0.9±0.7	2.1±1.9[a]	2.3±1.4[a,d]
Fasting glucose (mmol/L)	4.6±0.5	4.8±0.7	5.0±0.5	4.7±0.5	4.8±0.8
2-h OGTT glucose (mmol/L)	6.7±1.5	6.5±2.2	5.5±1.2	6.7±1.9	6.7±2.3
Fasting insulin (mIU/L)	2.0±1.5	10.4±8.6[b]	8.2±5.5[a]	11.7±8.3[a]	12.3±6.6[c]
HOMA-IR	0.9±0.8	2.3±0.2[b]	1.9±1.1[a]	2.4±1.5[a]	2.8±1.0[b,d]
ISI (Cederholm) (mg*L^2/mmol*mU*min) (25)	78.7±38.0	54.6±24.1[c]	73.4±54.6	51.0±17.1[c,f]	54.4±25.2[c,f]
Dysglycemia (n; %)	3 (6.9%)	17 (19.7%)[a]	0	3 (15%)	14 (25%)[a,d]

3.3.1 Circulating androgens and androgen excretion

Both serum A and T were significantly higher in PCOS than in healthy controls ($p<0.0001$ for both, Table 3-1). PCOS patients also had significantly higher FAI, DHEA and DHEAS (all $p<0.0001$) than controls. Total urinary androgen excretion as a reference marker of 24-h net production was also significantly higher in PCOS ($p<0.0001$, Table 3-2), with higher excretion of An ($p<0.001$), Et ($p<0.05$) and DHEA ($p<0.05$). T and A correlated strongly with FAI and total androgen metabolite excretion (Figure 3-1).

Serum A was elevated in 76 out of 86 (88.3%) PCOS patients, while T was elevated in 56 (65.1%). A was also elevated in 1/43 (2.3%) healthy controls; T was elevated in 5/43 (11.6%). Serum levels of A and T were in the normal range in 10 and 30 PCOS patients, respectively. Patients with PCOS were subsequently divided into four androgen phenotype groups dependent upon circulating androgens; normal A and concurrently normal T (NA/NT), normal A and high T (NA/HT), high A and normal T (HA/NT), and the most severe degree of androgen excess, high A and high T (HA/HT). Our analysis showed that the majority of patients fell into the HA/HT group (n=56), with 20 and 10 patients classified as HA/NT and NA/NT respectively; no patient had high T but normal A (Figure 3-2).

PCOS women in the group with the mildest degree of biochemical androgen excess (NA/NT) were similar to BMI-matched controls with regard to circulating DHEA, DHEAS, T, A, LH and the LH/FSH ratio. However they differed significantly from controls with regard to SHBG and FAI (Table 3-1). NA/NT PCOS women had androgen metabolite excretion rates similar to controls, while androgen excretion in the two other PCOS groups was significantly higher (Table 3-2, Figure 3-3). Similarly, serum DHEA and DHEAS were significantly higher in HA/HT and HA/NT patients as compared to the NA/NT group and the

controls (Figure 3-3). Clinically, the Ferriman-Gallwey score was significantly higher in HA/HT women compared to NA/NT (p=0.04) (Table 3-1).

Table 3-2 24-h urinary steroid excretion in PCOS patients, controls and PCOS androgen phenotype subgroups. Androgen and glucocorticoid metabolites were measured by gas chromatography/mass spectrometry. PCOS subgroup classification according to high (H) or normal (N) androstenedione (A) and testosterone (T) concentrations as measured by liquid chromatography/tandem mass spectrometry (normal T (NT) ≤1.9nmol/l; normal A (NA) ≤7.4nmol/l). Data expressed as mean±SD unless otherwise stated.

a, p<0.05, b, p<0.01, c, p<0.001 as compared to BMI-matched healthy controls

d, p<0.05; e, p<0.01; f, <0.001 as compared to NA/NT group

Variable	**Controls (n=43)**	**All PCOS (n=86)**	**NA/NT (n=10)**	**HA/NT (n=20)**	**HA/HT (n=56)**
Total androgen metabolites (µg/24h)	2882±2018	5493±2781[c]	2751±1760	4886±1968[a,d]	5727±2530[c,d]
An (µg/24h)	1413±906	2824±1651[c]	1452±1178	2594±1207[a]	3016±1553[c,d]
Et (µg/24h)	1468±1208	2457±725[c]	1299±733	2292±1211	2683±1469[a,e]
An/Et ratio (5α-reductase activity)	1.1±0.4	1.3±0.6[a]	1.2±0.6	1.3±0.6	1.3±0.6
Urinary DHEA(µg/24h)	457±721	1740±954[a]	477±400	1586±960	1960±854[a, d]
Total glucocorticoid metabolites (µg/24h)	6821±3572	9624±4214[b]	8449±4181	8935±3404	9803±4390[a]
5α-THF (µg/24h)	940±608	1313±814[a]	1250±1135	1259±759	1359±842[b]
THF (µg/24h)	1206±607	1520±549[a]	1489±924	1477±475	1634±822[a]
5α-THF/THF (5α-reductase activity)	0.8±0.3	0.9±0.5	0.8±0.4	0.8±0.5	0.9±0.5
(5α-THF+THF)/THE (11β-HSD1 activity)	0.9±0.3	0.9±0.3	0.9±0.3	0.9±0.2	0.9±0.4
Urinary cortisol/urinary cortisone (11β-HSD2 activity)	0.6±0.5	0.6±0.2	0.5±0.1	0.6±0.2	0.7±0.2

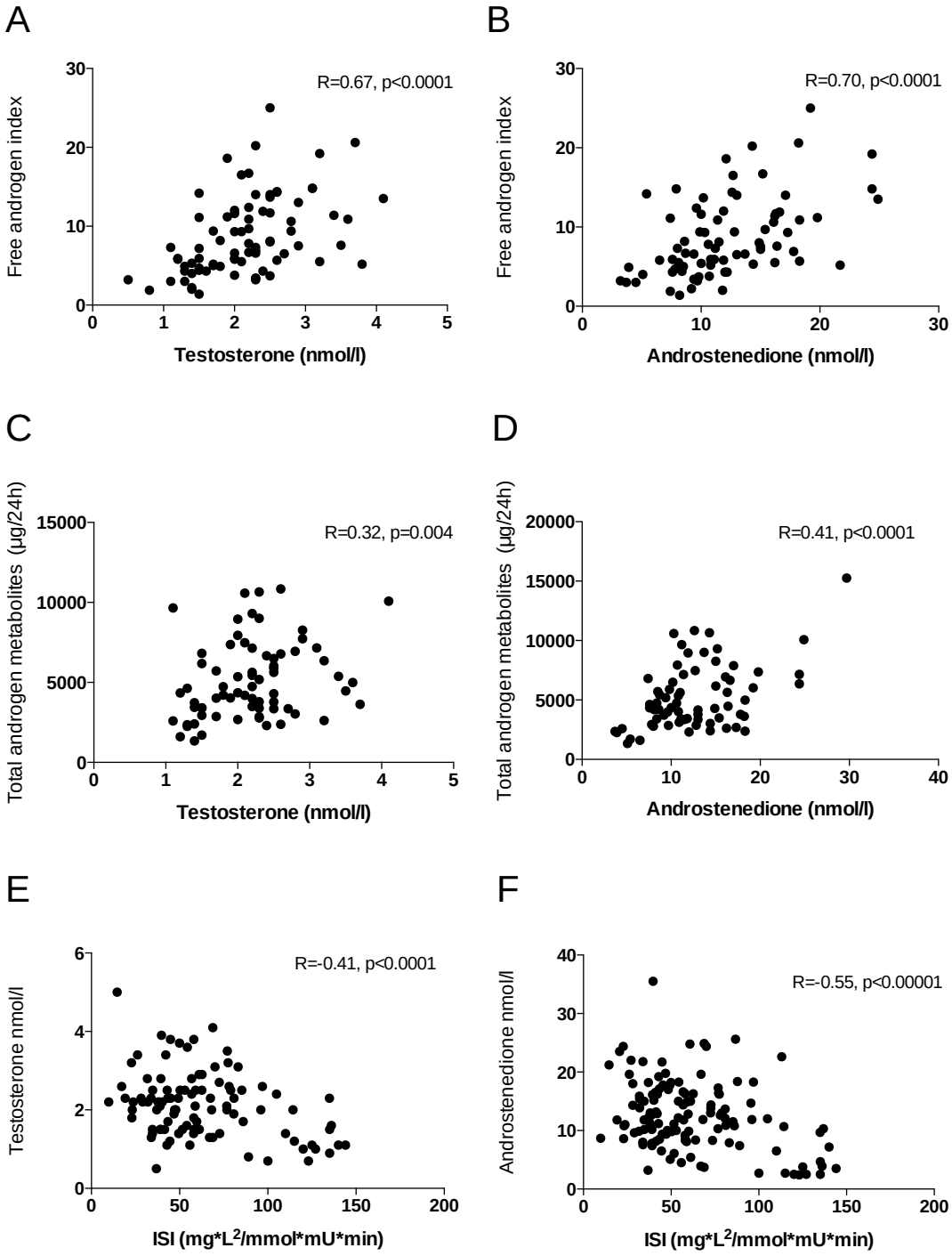


Figure 3-1 Relationship of serum testosterone (T) and androstenedione (A) with free androgen index (FAI), total urinary androgen metabolites and insulin sensitivity index (ISI).

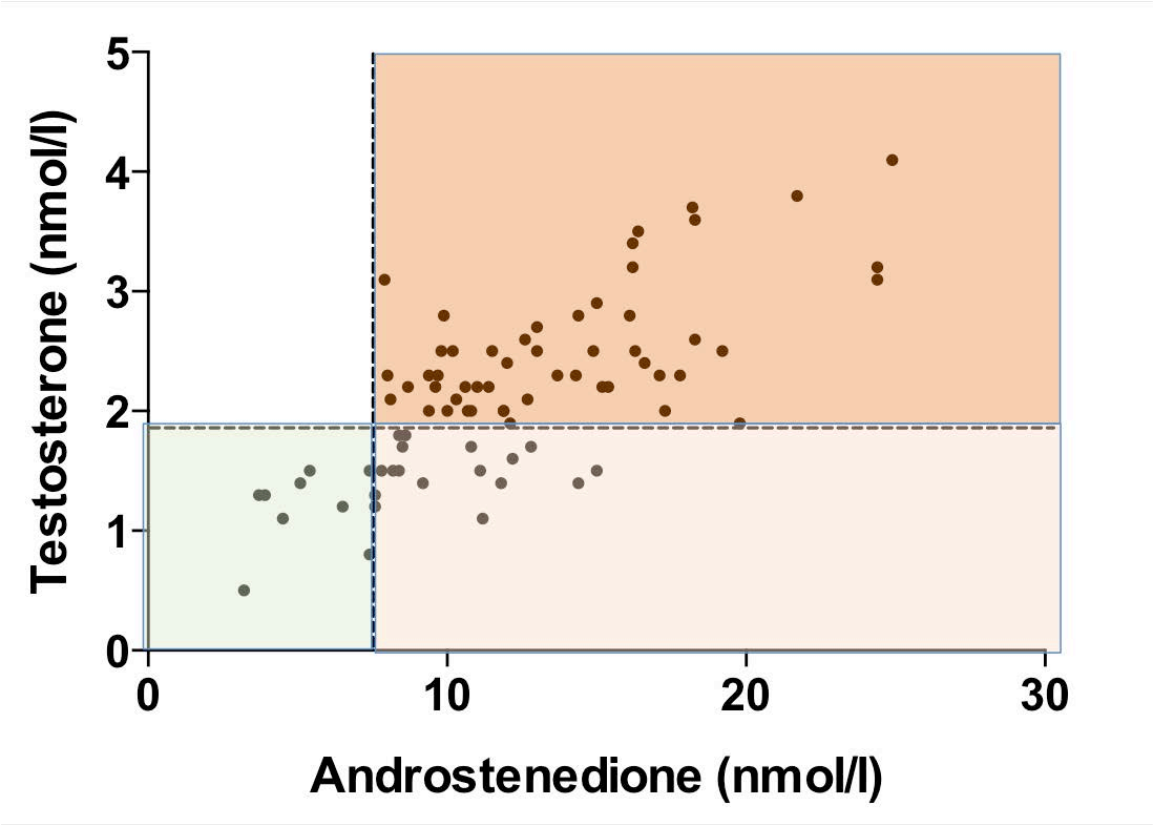


Figure 3-2 Relationship of serum testosterone (T) and androstenedione (A) with stratification of androgenemic subgroups: normal A and normal T (NA/NT, n=10); high A and normal T (HA/NT, n=20); high A and high T (HA/HT, n=56).

3.3.2 Metabolic characteristics

Overall, PCOS patients had higher fasting insulin and HOMA-IR values than BMI-matched controls but fasting glucose and 2-hour values did not differ significantly (Table 3-1). The incidence of dysglycemia was 19.7% in the PCOS group compared to 6.9% in controls (p=0.03). SHBG was significantly lower in the PCOS group compared to controls (p<0.001). No significant correlation was observed between BMI and T (R=0.14, p=0.13), A (R=0.01, p=0.91), or the T:A ratio (R=0.01, p=0.98). However, BMI was positively correlated with FAI (R=0.4, p<0.0001) and systemic 5α-reductase activity (An/Et, R=0.21, p=0.01; 5α-

THF/THF, R=0.20, p=0.02). T, A, and FAI all correlated positively with HOMA-IR (T: R=0.23, p=0.02; A: R=0.21, p=0.02; FAI: R=0.56, p<0.0001). ISI and HOMA-IS values were lower in PCOS patients compared to controls (p<0.001 for both). After correcting for age and BMI, ISI had a strong negative correlation with serum T and A (R=-0.41, p<0.001, and R=-0.55, p<0.00001, respectively, Figure 3-1)**.** When PCOS and control patients were considered separately, a negative association between A and ISI was observed in both groups (R=-0.53, p<0.00001, and R=-0.41, p<0.001, respectively). In obese control patients, this negative association between A and ISI persists (R=-0.25, p=0.04). On multiple linear regression analysis, a significant negative association was confirmed only between A and ISI (coefficient -2.69, 95% CI -1.57, -3.81, p<0.0001, Table 3-3); no such association was observed between ISI and either serum T, DHEAS or DHEA.

HOMA-IR in all three PCOS subgroups was significantly higher than in controls and HA/HT women had significantly higher values than NA/NT women (p<0.05 for both) (Figure 3-3). However, only HA/NT and HA/HT had lower ISI values (Table 3-1). PCOS women in the NA/NT group had significantly higher fasting insulin and HOMA-IR than BMI-matched controls but did not have any cases of dysglycemia, as documented by OGTT (Table 3-1). In contrast, 15% and 25% of women in the HA/NT and HA/HT groups, respectively, had an abnormal OGTT result. No significant differences were observed in systolic BP, TChol, HDL or TG between each group. In contrast to the significant differences found between the groups defined by A and T levels, no difference in insulin sensitivity was observed between the four PCOS phenotype groups defined by the presence of Rotterdam criteria (Figure 3-4).

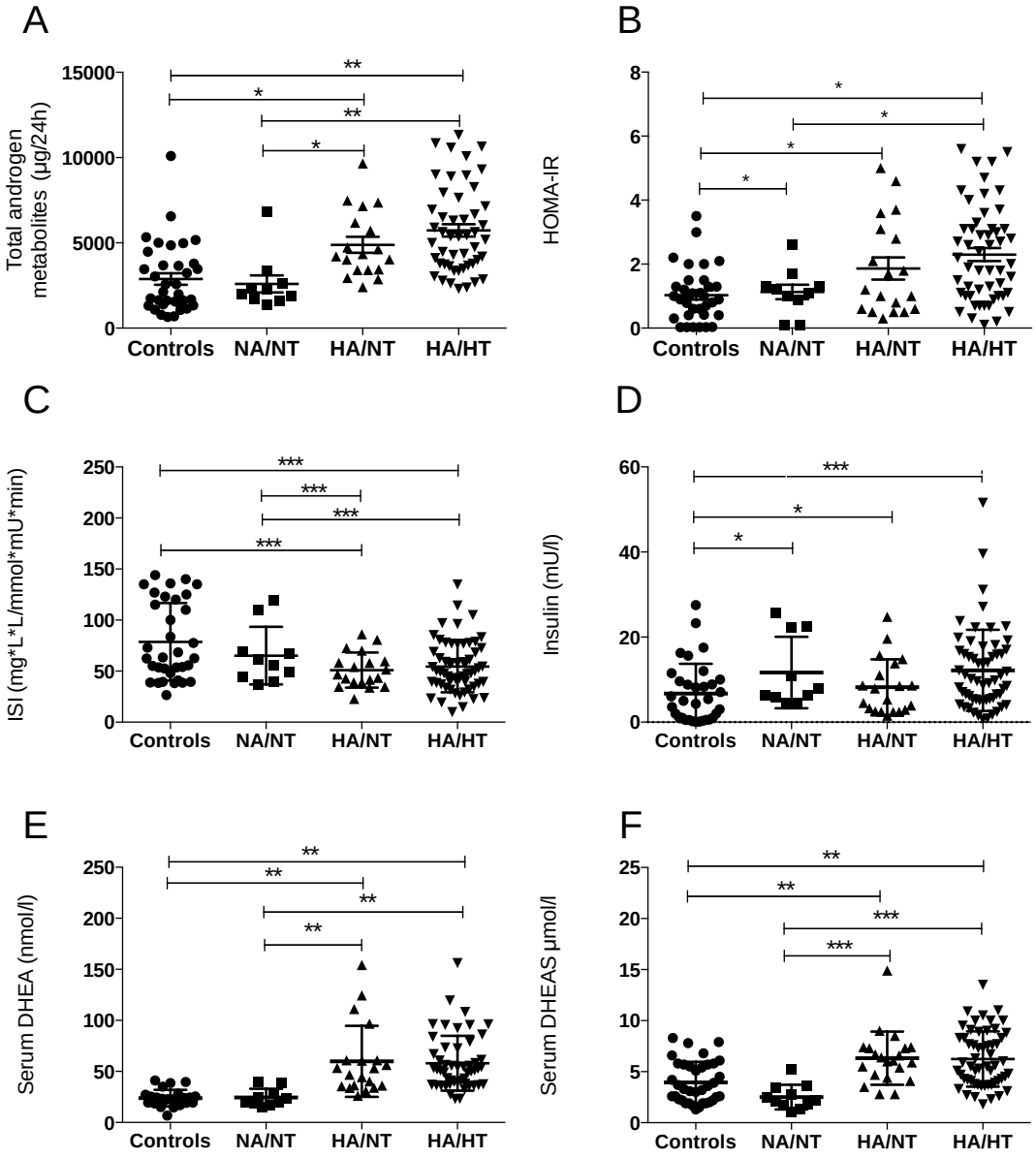


*Figure 3-3 Total androgen metabolites (A), HOMA-IR (B), ISI (C), fasting insulin (D), serum DHEA (E) and DHEAS (F) in the three androgen phenotype PCOS sub-groups and healthy controls. *p<0.05; **p<0.01; ***p<0.001.*

*Table 3-3 Multiple linear regression model for insulin sensitivity index (ISI). a, p<0.05; b, p<0.001, c, p<0.0011. [1]Represents unit change in ISI (mg*L^2/mmol*mU*min) for each unit increase in variable.*

Variable	**Coefficient[1]**	**95% CI (Upper, Lower)**
Age	-1.39[b]	-0.53, -2.24
BMI	-1.93[c]	-1.07, -2.80
Testosterone	6.58	18.79, -5.60
Androstenedione	-2.69 [c]	-1.57, -3.81
DHEA	-0.10	0.14, -0.34
LH	-0.61	0.06, -1.28
Total androgen metabolites	0.003	0.006, -0.001
Total GC metabolites	-0.003[a]	-0.001, -0.005

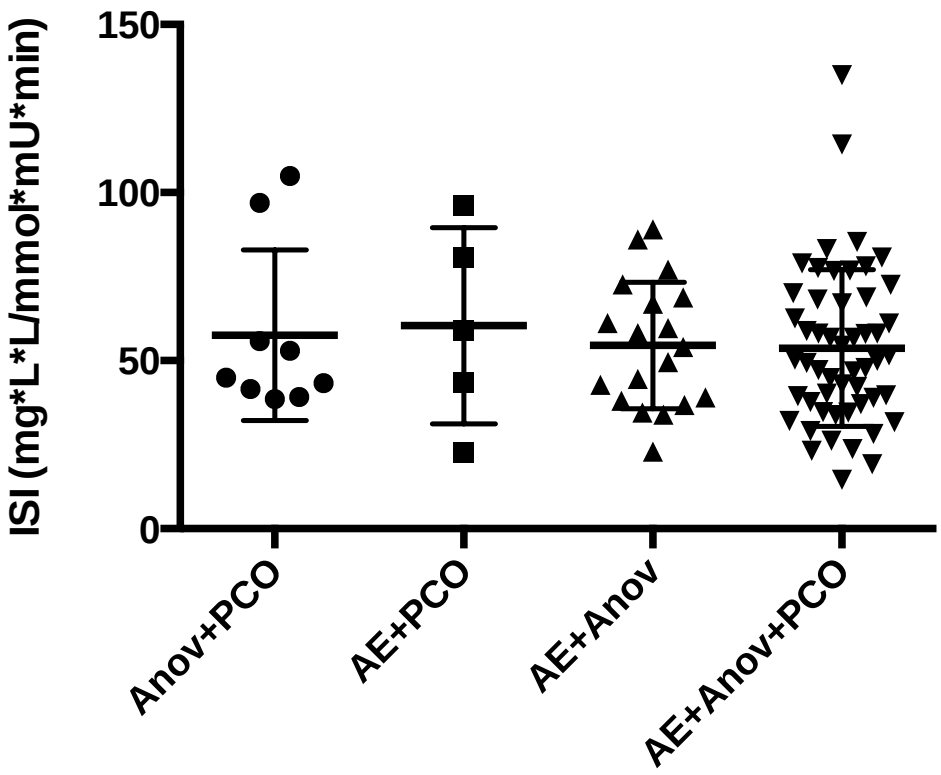


Figure 3-4 Insulin sensitivity index (ISI) in each of the four PCOS phenotype groups derived from the Rotterdam criteria.

3.3.3 Body composition

No significant differences in fat distribution or total lean mass were observed between PCOS and control women on DXA imaging. Similarly, there were no significant differences in body composition between the three PCOS androgen phenotype subgroups or the four PCOS phenotype groups. After controlling for age and BMI (partial correlation testing), the android:gynaecoid ratio was strongly correlated with HOMA-IR ($R=0.53$, $p<0.0001$), FAI ($R=0.37$, $p<0.0001$), An/Et ($R=0.38$, $p<0.0001$) , 5α-THF/THF ($R=0.31$, $p<0.0001$) and total glucocorticoid metabolites ($R=0.26$, $p=0.01$). There was also a strong negative correlation between the android:gynaecoid ratio and ISI ($R=-0.41$, $p<0.01$). Gynaecoid fat had a negative association with HOMA-IR ($R=-0.36$, $p=0.003$) and FAI ($R=-0.29$, $p=0.03$).

3.3.4 Cortisol metabolism

PCOS patients and controls did not differ significantly in indices of 11β-HSD1 or 11β-HSD2 activity (Table 3-2). However, total glucocorticoid excretion was higher in the PCOS group (9624±4214 *vs.* 6821±3572μg/24h, $p=0.01$). Total glucocorticoid metabolites correlated positively with HOMA-IR and negatively with ISI ($R=0.45$, $p<0.0001$, and $R=-0.43$, $p<0.0001$). On multiple linear regression (Table 3-3), total glucocorticoid metabolites were negatively associated with the ISI (coefficient -0.003, 95% CI -0.001, -0.005, $p=0.048$).

When carrying out a separate analysis for the PCOS androgen phenotype subgroups, urinary glucocorticoid excretion appeared to gradually increase with the degree of androgen excess, with significantly higher levels in the HA/HT group than in controls. Urinary levels of 5α-THF and THF were significantly higher ($p=0.005$ and $p=0.03$, respectively) in HA/HT women compared to controls.

3.4 Discussion

We have performed a detailed assessment of the clinical, metabolic and androgen phenotype of a cohort of PCOS, revealing that PCOS patients with co-elevation of A and T have impaired indices of insulin sensitivity compared to those with normal androgens or a solitary androgen elevation. In addition, using multiple linear regression, we found a strong negative relationship between A and insulin sensitivity as measured by the ISI, independent of age and BMI; such a relationship was not observed between T and ISI. All our patients were diagnosed according to consensus criteria and fulfilled at least two out of the three criteria oligo-/anovulation, polycystic ovaries on ultrasound and clinical signs of androgen excess, with other relevant etiologies excluded. Until relatively recently it has been unclear whether women with hyperandrogenic PCOS are at increased cardio-metabolic risk compared to those without clinical or biochemical signs of androgen excess. However, recent data suggest that biochemical androgen excess poses a higher risk of liver disease, insulin resistance and subclinical atherosclerosis compared to those with ovulatory dysfunction and polycystic ovaries alone (Jones et al., 2012, Barber et al., 2007). These findings suggest that hyperandrogenic PCOS represents a distinct metabolic phenotype, and that patients without androgen excess may have a phenotype similar to healthy controls (Dewailly et al., 2006). However, in our cohort we also found higher insulin and HOMA-IR values in normo-androgenemic PCOS compared to healthy controls, suggesting that women without androgen excess are not entirely protected from metabolic dysfunction.

Women with hyperandrogenic PCOS in our cohort had a significantly reduced ISI; those with normal serum androgens had ISI values comparable to healthy controls. However it is interesting that HOMA-IR and HOMA-IS values were still unfavorable in the NA/NT group, suggestive perhaps of impaired fasting indices but preserved ability to handle an oral

glucose load. Such an observation needs to be explored further and corroborated in future studies. Serum androgens, and in particular A, had a strong negative association with the ISI after correction for age and BMI, and this association was also observed in control patients with circulating A levels within the normal range. Overall these data lend support to the hypothesis that metabolic disease in PCOS should be considered as a continuous variable, with metabolic dysfunction observed across a worsening spectrum of severity of androgen excess. It is important to remember that serum cut-off values and normal ranges are relatively arbitrarily defined, and that androgen exposure across all patients with PCOS may contribute to their metabolic phenotype. Women with normal circulating androgens may still exhibit subtle hyperandrogenemia after LH stimulation (Gilling-Smith et al., 1997), which could explain the higher indices of insulin resistance observed. The long-term implications of this are unclear without longitudinal, prospective studies but these findings have also been observed by Barber and colleagues (Barber et al., 2007). When we subdivided our PCOS cohort into the four phenotypic groups arising from the Rotterdam consensus (Figure 3-4), no significant differences were observed in insulin sensitivity or body composition between each group. Therefore it is reasonable to argue that this strategy of sub-phenotyping of PCOS patients may not accurately predict those at highest metabolic risk. Interestingly Barber and colleagues found that patients in their AE+Anov+PCO subgroup were more insulin resistant; however, it is important to note that mean BMI of that group in the Barber study was significantly higher than in the other three phenotypic groups, whereas in our study the four groups were BMI-matched. For this reason we believe that co-measurement of A and T might offer a better alternative in terms of metabolic risk prediction than the previously suggested categorization by phenotype.

It is crucial that PCOS patients are correctly and accurately categorized as normoandrogenemic or hyperandrogenemic if they are to be stratified into low and high-risk metabolic groups on this basis. Our data show that measurement of serum T alone fails to adequately make this differentiation. Elaborate investigative tools such as LH stimulation testing or 24-h urinary androgen metabolite excretion as the reference standard for total androgen production are not universally available in routine clinical practice. By contrast, concurrent measurement of serum T and A provides a strategy that is easily applicable in clinical practice. In our view, only those with normal serum levels of both androgens should be categorized as normoandrogenemic; as discussed above, even this cohort may still exhibit occult hyperandrogenism, and without prospective studies it is impossible to be certain if they are protected from more severe metabolic disease over time. To date, there are only very limited data on the effects of androgen-lowering therapies on metabolic parameters in PCOS (Fruzzetti et al., 2010, Studen et al., 2011) and clearly larger prospective trials are needed.

Previous work has advocated measurement of a single morning serum testosterone sample during the early follicular phase as the diagnostic criterion for hyperandrogenemia (Barth et al., 2007). The limitations of total T as a solitary diagnostic tool for hyperandrogenemia have been described in the introduction of this paper and direct measurement of free T has been largely abandoned due to its notorious inaccuracy (Vermeulen et al., 1999), unless equilibrium dialysis is used, which is cumbersome, expensive and has very limited accessibility for routine clinical samples. The clearance and bioavailability of T are affected by serum SHBG levels, which are lowered by hyperinsulinemia, but A levels are not affected by this phenomenon (Anderson, 1974), and this may explain some of the observed discrepancies between T and A measurements. Appropriateness and reliability of analytical methods has equally been a major issue in this

debate, and the Endocrine Society has appealed to international laboratories to refine techniques for androgen measurement in women (Rosner et al., 2007). However the advent of LC-MS/MS has dramatically improved the rapid detection and reliable quantification of a range of serum steroids in both clinical and research practice (Cawood et al., 2005). Accurate A measurement could now emerge from these developments as a sensitive diagnostic test for hyperandrogenemia in PCOS.

Patterns of body fat distribution on DXA did not differ between PCOS and control patients, supporting previously published work (Barber et al., 2008). These and other studies point towards a qualitative rather than quantitative defect in adipose biology in PCOS (Barber and Franks, 2013). 17β-HSD5 is expressed in adipose tissue, and significant extra-ovarian conversion to testosterone may occur in obesity (Quinkler et al., 2004). Interestingly, there was also no difference in fat distribution phenotype between androgenemic subgroups in our cohort, and no correlation between BMI and serum androgens. 17β-HSD5 is also highly expressed in the ovary, and activating variants result in a PCOS phenotype with severe insulin resistance (Qin et al., 2006). This suggests a possible primary role for androgen excess in insulin resistance. However, the prevalence of increased insulin resistance in patients with elevated T and A in our study could also point towards an insulin-mediated effect on ovarian thecal steroidogenesis. The observation that insulin plays a key role in thecal androgen generation *in vivo* and *in vitro* supports this hypothesis (Cara and Rosenfield, 1988, Tosi et al., 2012). Insulin-stimulated A generation from the ovarian theca may create an enlarged circulating pool for conversion to T, and elevated levels of the former may therefore be a surrogate marker for metabolic dysfunction.

One limitation of our study is its cross-sectional nature. Longitudinal studies will reveal whether initial increases in androgen precursors later followed by additional increases

in T may represent a sequence observed over time in individual patients. We also accept that HOMA-IR measurement is not the gold-standard test assessment of tissue-specific insulin sensitivity. However the ISI devised by Cederholm and calculated in our cohort has been shown to perform with comparable accuracy to a hyperinsulinemic euglycemic clamp (approx. R=0.8) (Cederholm and Wibell, 1990, Stumvoll et al., 2000). Similarly, it was not possible to measure serum androgens in the early follicular phase of all patients, largely due to the fact that most PCOS patients were oligo- or amenorrhoeic. Finally the debate over what constitutes normal androgen values continues, and quoted reference ranges refer to normative values in 95% of an unselected presumptively healthy female population, implying that 'abnormal' levels will be observed in 5% of women with no evidence of endocrinopathy.

In conclusion, these data endorse the hypothesis that serum A is a more sensitive indicator of PCOS-related androgen excess that serum total T concentrations. We found that increasing androgen burden is associated with an adverse metabolic phenotype, and concurrent measurement of both A and T has highlighted a cohort of PCOS women that appears to be at increased metabolic risk. The utility of this novel tool for predicting metabolic risk in PCOS women warrants investigation in prospective and longitudinal studies and these may ultimately afford a personalized approach to the management of PCOS. The next challenge is to tease out the specific contribution of adipose tissue to androgen generation in PCOS, and how local androgen exposure adversely impacts on the metabolic phenotype. These will be explored in chapters 4 and 5, respectively.

4 AKR1C3 expression and activity in human adipose tissue

4.1 Introduction

Hyperandrogenism in PCOS has classically been attributed to increased ovarian and adrenal secretion of androgen precursors and active androgen metabolites (Franks, 1995). However, recent focus has increasingly shifted to peripheral tissues, which are not only targets of androgen action, but also play an important role in pre-receptor metabolism and androgen activation (Labrie et al., 2003). This process of intracrine activation is discussed in-depth in section 1.4.2. Adipose tissue is a key peripheral organ of androgen activation and secretion (Blouin et al., 2009a). Evidence for the role of adipose tissue in systemic androgen metabolism is highlighted in studies showing positive correlations between circulating sex steroid levels, BMI and fat mass in females (Mongraw-Chaffin et al., 2015, Borruel et al., 2013). These associations are complex and may exhibit sexual dimorphism; obesity in men is associated with low circulating sex steroid levels and hypogonadism (Mongraw-Chaffin et al., 2015). However, it is increasingly clear that androgen excess in females is intimately connected to both total adiposity and fat distribution, and may represent a component of the metabolic syndrome in premenopausal women (Corbould, 2008).

As discussed in section 1.7.2, adipose tissue has a complex network of androgen activating and inactivating enzymes (O'Reilly et al., 2014a). Important androgen-activating enzymes include 5αR1 and AKR1C3. 5αR1 converts T to the more potent DHT. AKR1C3 has predominant 17β-HSD activity, converting A into T, and is also referred to as 17β-HSD type 5. Expression and activity were correlated with body mass index in one small human study, and weight loss resulted in significant downregulation of mRNA expression (Quinkler et al., 2004). AKR1C3 is also highly expressed in the ovaries and adrenals, and activating polymorphisms are associated with an increased risk of PCOS (Ju et al., 2015). Adipose tissue

is also capable of androgen inactivation; AKR1C2 (also called 3α-HSD3) converts DHT into the weak 3α-diol through its 3α-reductase activity (Blouin et al., 2003). It is believed that androgen activation and inactivation are delicately balanced in adipose tissue, but factors influencing this equilibrium are poorly understood.

Severity of hyperandrogenism in PCOS is closely correlated with obesity, insulin resistance and circulating insulin levels (Nestler and Jakubowicz, 1997). The effects of insulin on ovarian hyperandrogenism are well documented *in vivo* and *in vitro* (Dunaif and Graf, 1989, Poretsky et al., 1999), and are summarized in Table 1-4. Insulin acts in synergy with pituitary gonadotrophins to increase ovarian thecal androgen generation. It drives local LH receptor recruitment, and directly stimulates thecal 17α-hydroxylase activity (Nestler et al., 1998). Systemically, insulin is correlated with total body 5α-reductase activity (Vassiliadi et al., 2009, Tsilchorozidou et al., 2003), implying a significant role in global rather than organ-specific androgen metabolism. This relationship is observed independent of BMI. The role of insulin in adipose androgen metabolism has not been delineated to date. Insulin-responsive transcriptional regulators of AKR1C3 have recently been characterized (Du et al., 2009), raising the exciting possibility of a direct role for insulin in adipose androgen activation. As discussed above, obesity *per se* may also play an important role in androgen metabolism; most studies have found a positive association between T and adiposity in women, but the converse appears to be the case in men (Mongraw-Chaffin et al., 2015, Oh et al., 2002). Whilst fat mass itself may drive adipose androgen generation, some of the observed effects in PCOS might also be mediated by obesity-induced hyperinsulinaemia.

We hypothesise that hyperinsulinaemia in PCOS drives adipose androgen activation in a similar manner to that observed in the ovary. We also hypothesise that mRNA expression of androgen-activating enzymes in adipose tissue is closely associated with body mass index in

female patients. In order to test these hypotheses, I will measure mRNA expression of enzymes of interest in adipose tissue explants obtained from male and female subjects, across both subcutaneous and omental fat depots, and correlate expression levels with age and BMI. Furthermore, I will examine the role of insulin in adipose androgen metabolism in a human adipocyte cell line, SGBS, and validate findings where possible in primary differentiated female OM and SC adipocytes. Cells will be exposed to hyperinsulinaemic conditions *in vitro,* with measurement of AKR1C3 mRNA expression by realtime-PCR, and determination of functional activity by measurement of conversion of A to T by LC-MS/MS.

4.2 Methods

4.2.1 Research strategy

In this chapter, I will study mRNA expression of the androgen-activating enzymes AKR1C3 and 5αR1, and the inactivating enzyme AKR1C2, in adipose tissue explants from both male and female patients undergoing abdominal surgery at the Queen Elizabeth Hospital Birmingham. Expression levels will be examined across SC and OM fat depots, and correlated with age and BMI in both sexes. Furthermore, I will examine the SGBS preadipocyte cell line as a valid representative model for adipose androgen metabolism in humans. mRNA expression of key markers of adipogenesis and lipid metabolism will be examined across differentiation (days 0, 7 and 14). Androgen receptor (AR), AKR1C3 and SRD5A1 expression will also be determined to validate the utility of the SGBS model as a tool to study adipose androgen metabolism.

In order to study the effects of hyperinsulinaemia on androgen generation from adipocytes *in vitro,* differentiated SGBS cells will be exposed to incremental doses of insulin. Realtime PCR will be used to examine the effect of insulin exposure on AKR1C3 mRNA expression. Additionally, I will employ LC-MS/MS as a functional tool to study adipocyte AKR1C3 activity under normal and hyperinsulinaemic conditions, by measuring T generation after incubation with A. Findings will be replicated and validated where possible in primary human OM and SC cultures differentiated *ex vivo.* Finally, pharmacological inhibition of AKR1C3 and of the insulin-stimulated PI3K pathway will be employed to determine if local androgen metabolism can be ameliorated by selective modulation of the androgen and insulin metabolic pathways.

4.2.2 Subjects

Local research ethics committee (REC) approval was obtained. Patients undergoing abdominal surgery at the Queen Elizabeth Hospital gave written informed consent in order to provide paired OM and SC fat biopsies to the Human Biorepository at the University of Birmingham. Exclusion criteria included pregnancy, diabetes, glucocorticoid treatment, and inflammatory or neoplastic disease. Samples obtained were used for primary cell culture experiments (section below), or were dissected into whole tissue explants. These explants were stored in RNALater at -20°C until RNA extraction.

4.2.3 SGBS cell culture

Proliferating SGBS cells were seeded in 12- or 24-well plates and grown to confluence in DMEM-F12 with 10% FCS, additionally supplemented with 33μM biotin, 17μM pantothenate and penicillin-streptomycin 1% (growth media). In preadipocyte studies, cells were exposed to treatments, and biomaterial extracted, before differentiation. In adipocyte studies, SGBS cells were differentiated over 14 days in OF media, which was further supplemented according to the differentiation protocol described in section 2.2. All cells were cultured in serum-media (OF only without additives) for 24 hours before experiments.

4.2.4 Human preadipocyte cell culture

Primary human fat samples were obtained as described in section 4.2.2, and extracted from whole OM and SC tissue according to the protocol described by Bujalska *et al* (Bujalska et al., 1999) and summarized above in section 2.1. Briefly, adipose tissue samples were

digested with type 2 collagenase at 37°C for one hour, followed by sterile filtration through layered gauze to remove solid components. After centrifugation, the stromovascular pellet containing preadipocytes was resuspended in media and plated in 12- or 24-well plates. Preadipocytes were grown to 80% confluence before differentiation into mature adipocytes over 14 days in chemically-defined media. All treatment protocols involved use of serum-free media without additives for 24 hours.

4.2.5 Cell treatments

All treatments were diluted as appropriate in serum-free media. AKR1C3 activity assays were performed by the addition to A to wells containing serum-free media for 4, 8 or 24 hours (as appropriate to the experiment). The AKR1C3 inhibitor, 3-4-trifluoromethyl-phenylamino-benzoic acid (Merck Millipore, UK), was added to wells for 15 minutes at a concentration of 500nM before addition of A. The PI3K inhibitor wormannin (500nM) was added 15 minutes before addition of insulin in selected experiments.

4.2.6 RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted using the Tri-Reagent protocol (section 2.4.1). RNA concentration and purity were assessed using NanoDrop technology as described above. One microgram of total RNA was used for reverse transcription (see section 2.4.2); cDNA was diluted 1:10 with RNase-free water. Real-time PCR was used to determine adipose mRNA expression of the key adipogenic markers LPL and PPAR-γ, components of the insulin-signalling pathway (PI3K and IRS-2), and crucial regulators of androgen metabolism (AR, AKR1C3, SRD5A1 and SRD5A2). Real-time PCR reactions were performed as described in

section 2.4.3. mRNA levels were detected using an ABI 7500 sequence detection system (Applied Biosystems/Life Technologies, Paisley, UK). All probes and primers were supplied by Applied Biosystems/Life Technologies as expression assays (Taqman probe/primer mix). All reactions were normalized against two housekeeping genes, 18S and GAPDH.

4.2.7 Cell media steroid extraction and LC-MS/MS analysis

Steroids were extracted for LC-MS/MS analysis of AKR1C3 activity as described in section 2.5.2. Briefly, A substrate (200nM) was added to wells of SGBS cells or primary differentiated adipocytes containing serum-free media without additives. After 4, 8 or 24 hours (as appropriate to each experiment), media was removed and placed in sylinised glass TLC tubes, to which 20µl internal standard was added. A calibration series was prepared for each experiment as described in Table 2-1. MTBE (3ml) was added to each sample, followed by freezing (1-24 hours). After evaporation, samples were reconstituted in 125µl of methanol:H_20 (50:50) and frozen at -20°C until ready for LC/MS-MS analsysis. AKR1C3 activity was determined by the generation of T from A substrate over a defined time period. Comparison for T generation between treatments and controls was performed by normalizing control data to 100%.

4.2.8 Statistical analysis

Normally distributed data were analysed using unpaired student *t* tests to compare single treatments to controls; non-parametric tests were used when normality tests failed. When comparing means from multiple groups, one-way ANOVA with post-hoc Tukey testing was employed. All statistical analysis on realtime PCR data was performed for minimum of n=3 on Δct values between different treatment groups. Arbitrary mRNAunits were log-transformed. Correlation testing was employed to examine linear relationships between continuous variables, using Pearson's test (parametric) or Spearman's rank test (non-parametric data) as appropriate.

4.3 Results

4.3.1 Androgen-metabolising enzyme mRNA expression in male and female adipose tissue explants

4.3.1.1 Subjects

Paired SC and OM fat samples were obtained from 66 participants (26 male). Baseline demographic data is shown in Table 4-1. No significant differences were observed in age and BMI between male and female patients (p=0.36 and p=0.45, respectively).

Table 4-1 Baseline characteristics of study group

Variable (mean±SEM, range)	**Male (n=26)**	**Female (n=40)**
Age (years)	61.6±2.8 (28-82)	58.3±2.2 (27-84)
BMI (kg/m^2)	27.6±0.8 (20.7-37.4)	28.6±1.0 (21.3-53.3)

No significant differences were observed in SC and OM mRNA expression of AKR1C3, AKR1C2 or SRD5A1 between male and female patients, Table 4-2.

Table 4-2 Comparison of mRNA expression of androgen-metabolising enzymes between males and females in SC and OM adipose tissue (data expressed as mean ∆ct±SEM)

Gene	**Male (n=26)**	**Female (n=40)**	**P-value**
AKR1C3 SC	7.5±1.0	7.3±0.7	0.83
AKR1C3 OM	9.5±0.6	9.2±0.6	0.67
AKR1C2 SC	19.5±0.9	20.3±0.9	0.57
AKR1C2 OM	19.3±1.5	21.2±0.5	0.14
SRD5A1 SC	11.9±0.7	11.8±0.7	0.95
SRD5A1 OM	12.7±0.5	12.4±0.4	0.71

4.3.1.2 Depot-specific differences

When male and female patients were analysed together, AKR1C3 expression was significantly higher in SC compared to OM fat (mean Δct 7.1±0.6 v 9.2±0.4, p=0.004, Figure 4-1). Expression of AKR1C2 and SRD5A1 did not differ significantly between SC and OM tissue (20.6±0.6 v 19.3±0.8 and 12.8±0.4 v 11.8±0.5, p=0.22 and p=0.09, respectively). In male patients, no differences in SC compared to OM mRNA expression of AKR1C3, AKR1C2 or SRD5A1 were observed (7.5±1.0 v 9.5±0.6, p=0.10, 19.5±1.0 v 19.3±1.5, p=0.89, and 11.9±0.7 v 12.7±0.5, p=0.35, respectively). However, in female patients, AKR1C3 expression was significantly higher in SC compared to OM fat (7.3±0.7 v 9.2±0.6, p=0.04).

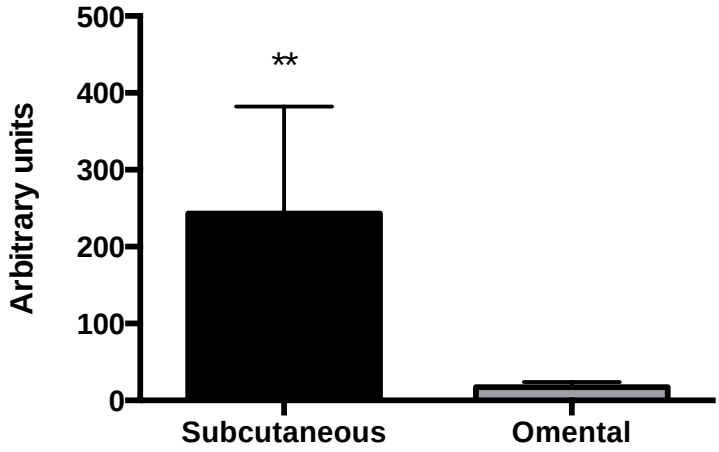


*Figure 4-1 Relative AKR1C3 mRNA expression in SC and OM adipose tissue (all patients, n=66). **p<001 for ∆ct.*

4.3.1.3 Associations between androgen gene expression, age and BMI

Subject samples were next categorized into those from patients aged <55 years and those ≥55 years. Expression of AKR1C3, AKR1C2 and SRD5A1 in OM fat did not differ significantly between the two age categories (Table 4-3). However, AKR1C3 expression was significantly higher in SC fat in patients aged <55 years compared to those aged 55 years or above (5.6±1.4 v 8.2±0.5, p=0.04, Table 4-3).

Table 4-3 mRNA expression of androgen-metabolising enzymes according to age category in SC and OM adipose tissue (data expressed as mean ⊿ct±SEM)

Gene	**Age <55 years (n=19)**	**Age≥55 years (n=47)**	**P-value**
AKR1C3 SC	5.6±1.4	8.2±0.5	0.04*
AKR1C3 OM	8.6±0.8	9.6±0.4	0.24
AKR1C2 SC	19.2±1.5	20.3±0.6	0.45
AKR1C2 OM	21.5±0.6	20.3±0.8	0.41
SRD5A1 SC	10.6±0.9	12.4±0.6	0.09
SRD5A1 OM	12.8±0.7	13.0±0.5	0.88

Samples were further categorized into those obtained from non-obese (BMI<29.9kg/m^2) and obese patients (BMI≥30.0kg/m^2). Differences in mRNA expression of key genes between obese and non-obese patients are shown in Table 4-4 for SC and OM fat.

Table 4-4 mRNA expression of androgen-metabolising enzymes according to BMI category in SC and OM adipose tissue (data expressed as mean ⊿ct±SEM)

Gene	**Non-obese (n=49)**	**Obese (n=17)**	**P-value**
AKR1C3 SC	8.1±0.7	5.3±1.2	0.04*
AKR1C3 OM	9.8±0.4	8.0±0.9	0.06
AKR1C2 SC	20.6±0.5	18.2±1.7	0.10
AKR1C2 OM	20.6±0.6	20.6±1.5	0.97
SRD5A1 SC	12.1±0.5	11.1±1.1	0.38
SRD5A1 OM	13.0±0.5	12.8±0.9	0.84

No significant differences in mRNA expression of AKR1C3, AKR1C2 or SRD5A1 were observed in SC or OM adipose tissue samples between non-obese and obese male patients. In female OM adipose tissue, expression of our genes of interest did not differ significantly between obese and non-obese patients. However, in female SC adipose tissue, AKR1C3 expression was significantly higher in obese compared to non-obese patients (4.2±1.9 v 8.2±0.6, p=0.02). Expression of AKR1C2 (17.8±3.0 v 21.0±0.7, p=0.13) and SRD5A1 (10.9±1.7 v 12.1±0.7, p=0.51) in female SC tissue was similar in obese and non-obese patients.

Using correlation testing, we next examined the relationship of AKR1C3, AKR1C2 and SRD5A1 expression with age and BMI. In male OM tissue, no associations were observed between gene expression and age or BMI. In male SC, expression of AKR1C3 was negatively associated with age (R=0.65 for Δct, p=0.003). In female OM fat, no associations were observed between gene expression and age. OM AKR1C3 expression was positively associated with BMI (R= -0.41, p=0.025). The relationships between age, BMI and gene expression in female SC fat are summarized in Table 4-5. AKR1C3 expression had correlated positively with BMI (Figure 4-2), as well as with expression of AKR1C3 and SRD5A1. AKR1C2 expression was also positively associated with BMI.

*Table 4-5 Relationship of age and BMI with mRNA expression (Δct) of androgen-metabolising genes (R-value) in female SC adipose tissue. *p<0.05; **p<0.01; ***p<0.001.*

	Age	**BMI**	**AKR1C3**	**AKR1C2**	**SRD5A1**
Age	-	-0.25	-0.22	0.23	0.12
BMI	-0.25	-	-0.51*	-0.71**	-0.10
AKR1C3	-0.22	-0.51*	-	0.71**	0.72**
AKR1C2	0.23	-0.71**	0.71**	-	0.83**
SRD5A1	0.12	-0.10	0.72**	0.83**	-

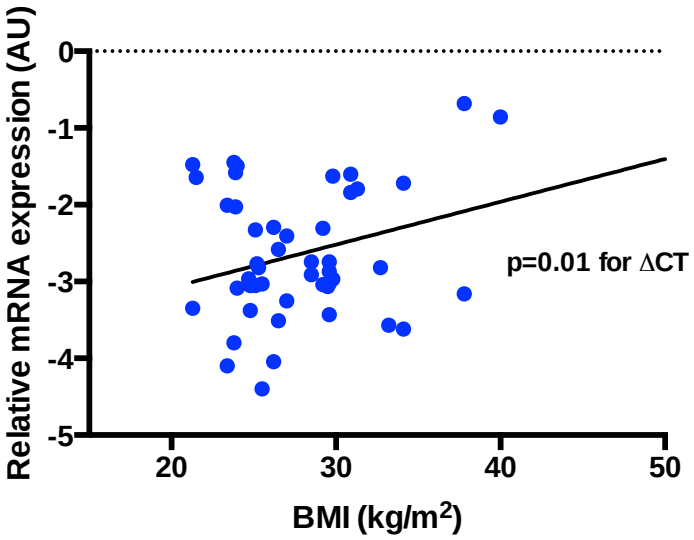


Figure 4-2 Postive relationship between BMI and relative AKR1C3 mRNA expression (log-transformed) in female SC adipose tissue (n=38; Pearson's correlation testing for BMI and AKR1C3 ∆ct)

4.3.2 Validation of SGBS cells as model of human adipocyte and adipose androgen metabolism

4.3.2.1 Lipid accumulation

Morphological changes were observed across differentiation in SGBS cells over 14 days. Cytoskeletal changes resulted in loss of fibroblast appearances. Cells also became more round in shape, and intracellular lipid accumulated consistent with differentiation into adipocytes (Figure 4-3).

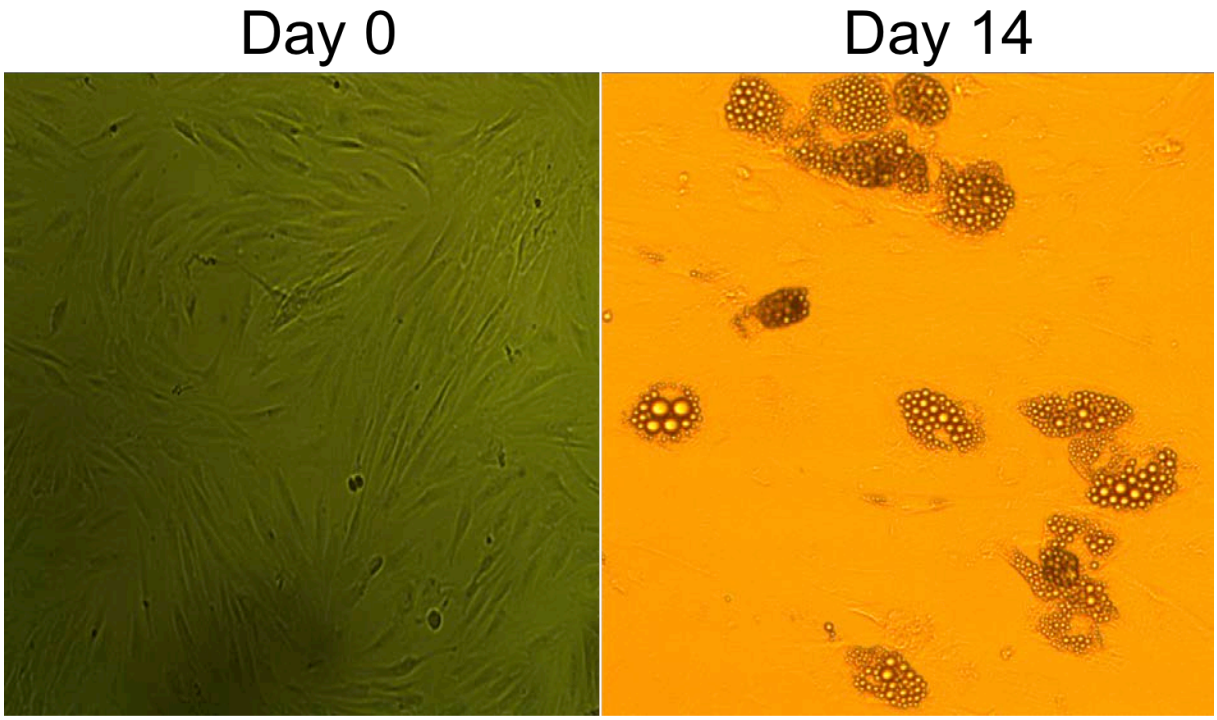


Figure 4-3 SGBS cells as confluent preadipocytes (day 0) and after differentiation process for 14 days in chemically-defined medium (magnification x10).

4.3.2.2 Markers of differentiation, androgen metabolism and insulin signaling cascade in SGBS cells

Using real-time PCR, we compared mRNA expression of key markers of adipocyte differentiation, androgen metabolism and the insulin signalling cascade across differentiation in SGBS cells, from Day 0 (preadipocyte) to Day 14 (adipocyte). Significant upregulation in mRNA expression of almost all key component genes was observed between Day 0 and Day 14 (Table 4-6, Figure 4-4).

Table 4-6 Changes in mRNA expression of markers of adipocyte differentiation, androgen metabolism and the insulin signalling cascade across SGBS differentiation (data expressed as mean ∆ct±SEM for n=3-5 experiments)

Gene	SGBS Day 0 (preadipocyte)	SGBS Day 7	SGBS Day 14 (adipocyte)	P-value (Day 0 v 14)
PPAR-γ	7.2±0.8	3.8±0.3	4.0±0.2	0.03
LPL	Undetectable	8.3±0.5	6.3±0.1	<0.0001
AR	18.8±0.7	16.8±0.9	15.4±0.4	0.02
AKR1C3	13.3±0.1	8.8±0.7	7.9±0.9	0.02
AKR1C2	23.5±0.9	-	19.1±0.4	0.01
SRD5A1	10.6±0.5	7.8±0.3	8.3±0.3	0.02
IRS-2	12.3±1.2	12.5±0.2	10.4±0.6	0.03
PI3Kca	16.9±0.5	17.1±0.2	14.7±0.8	0.04
ACC1	6.5±0.6	-	5.6±0.3	0.19
ACC2	9.5±0.4		5.9±0.5	0.001
FAS	5.0±0.2	-	4.1±0.3	0.04
FABP4	17.1±0.3	-	3.8±0.4	<0.0001

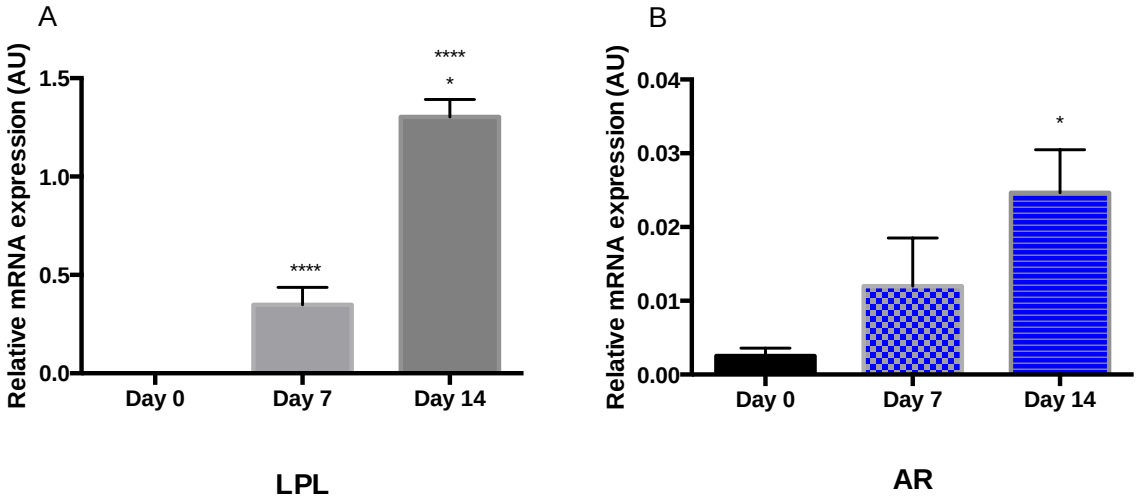


*Figure 4-4 Relative mRNA expression across SGBS differentiation of (a) lipoprotein lipase (LPL) and (b) androgen receptor (AR). *p<0.05; ****p<0.0001 for ∆ct compared to Day 0.*

4.3.3 AKR1C3 expression and activity in SGBS cells

4.3.3.1 AKR1C3 expression and activity across differentiation

AKR1C3 mRNA expression increased significantly across SGBS differentiation between Day 0 and Day 14 (mean Δct at Day 0 and Day 14 13.3±0.1 v 7.9±0.9, respectively, p=0.02, Figure 4-5).

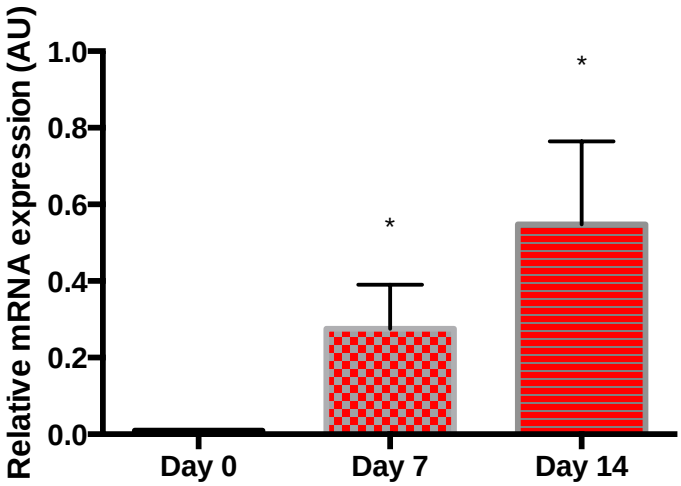


*Figure 4-5 Relative AKR1C3 mRNA expression in SGBS cells across differentiation. *p<0.05 compared to Day 0.*

AKR1C3 activity, determined by the generation of T from 200nM A over 24 hours, was significantly higher in differentiated adipocytes compared to preadipocytes (7.9±2.7 v 0.9±0.1 nM/24 hours, p=0.01, Figure 4-6).

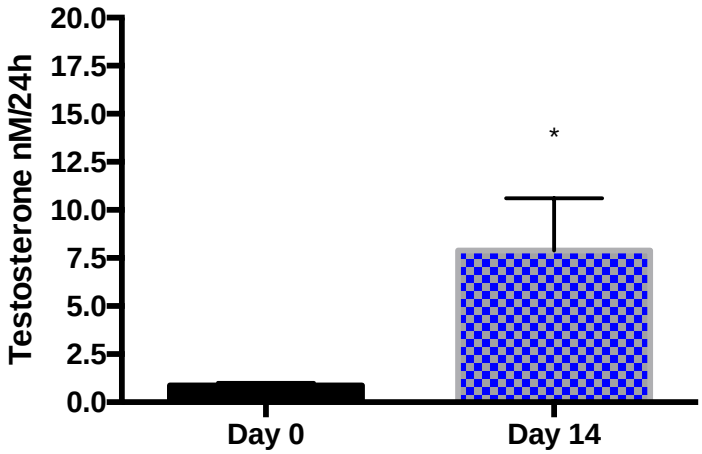


*Figure 4-6 AKR1C3 activity as determined by testosterone generation from androstenedione (200nM) over 24 hours in undifferentiated and differentiated SGBS cells. *p<0.05.*

T generation was significantly higher in SGBS cells incubated with A for 24 hours compared to those incubated for 4 and 8 hours (16.2±3.1 v 2.4±0.2 and 3.5±0.3nM respectively, p=0.01 for both, Figure 4-7).

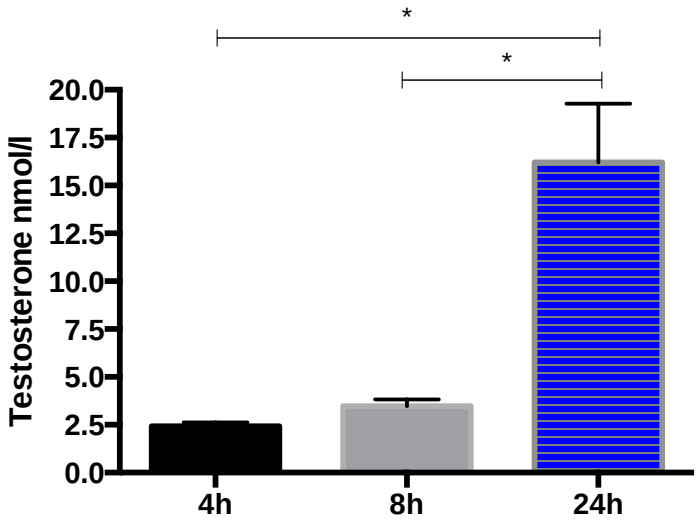


*Figure 4-7 Time course of AKR1C3 activity in differentiated SGBS cells at 4, 8 and 24 hours.*p<0.05.*

4.3.3.2 Effect of insulin on AKR1C3 expression and activity in SGBS cells

Incubation of SGBS cells with 10nM insulin had a trend towards increasing AKR1C3 expression but this effect did not reach statistical significance (mean Δct control 9.9±0.6 v 10nM insulin 7.3±0.1, p=0.057). However, incubation with 20nM insulin significantly increased AKR1C3 expression compared to both control and 10nM treatment (mean Δct 6.5±0.1 v 9.9±0.6 and 7.3±0.1, p=0.03 and 0.02, respectively, Figure 4-8).

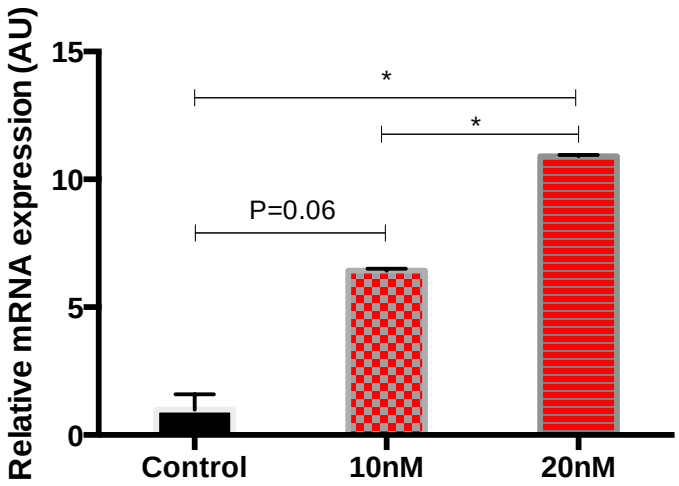


*Figure 4-8 Effect of insulin on AKR1C3 mRNA expression in SGBS cells. *p<0.05.*

AKR1C3 activity, as determined by generation of T after 24-hour incubation with A 200nM, increased significantly after exposure of SGBS cells to insulin (100% [control] v 131±9.8% [10nM], p=0.01; 100% [control] v 161.3±11.3% [20nM], p=0.0006, Figure 4-9). AKR1C3 inhibition significantly reduced T generation in both control adipocytes and those treated with 10nM insulin (100% [control] v 58.6±2.5% [inhibitor], p<0.0001; 131±9.8% [10nM] v 76.9±15.5% [inhibitor], p=0.02). Inhibitor treatment of cells exposed to 20nM insulin had a trend towards reducing T generation compared to insulin alone but this did not

reach statistical significance (161.3±11.4% [20nM] v 112.5±10.2% [inhibitor], p=0.08, Figure 4-9).

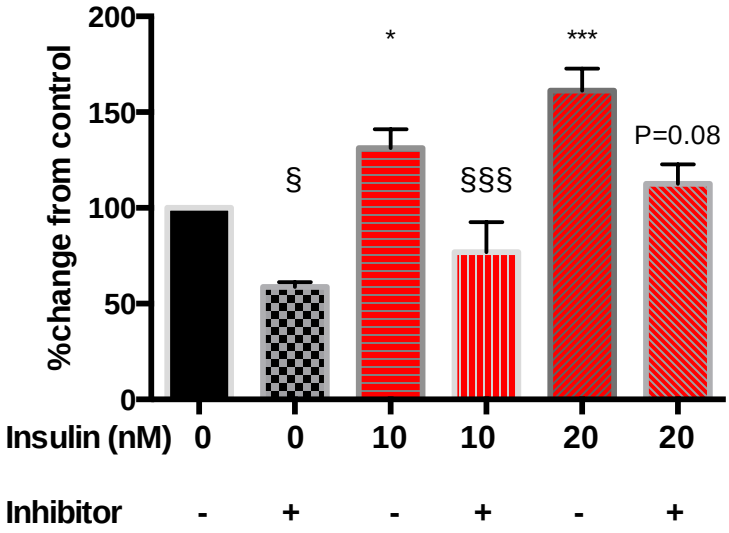


*Figure 4-9 Effect of insulin and AKR1C3 inhibition with 3-4-trifluoromethyl-phenylamino-benzoic acid (Merck Millipore, UK) on T generation in SGBS cells. Differentiated adipocytes were treated with and without insulin (10 or 20nM) and AKR1C3 inhibitor (500nM) for 24 hours. Insulin-stimulated increases in adipocyte testosterone generation are reduced by AKR1C3 inhibition in cells treated with 10nM insulin, although there is only a trend towards significant reduction in cells treated with 20nM insulin + inhibitor. Data represented as mean±SEM % change from control (100%); *p<0.05 vs control; ***p<0.001 compared to control; § p<0.05 for inhibitor vs corresponding treatment.*

Treatment of control and insulin-treated adipocytes with the PI3K inhibitor wortmannin had no significant impact on T generation in SGBS cells, Figure 4-10).

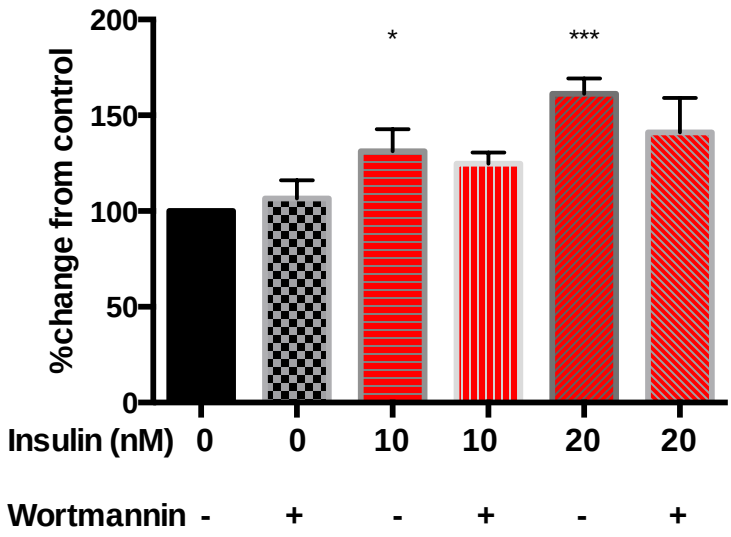


*Figure 4-10 Effect of insulin and wortmannin (500nM) on T generation in SGBS cells. *p<0.05 compared to control; ***p<0.001 compared to control.*

4.3.4 AKR1C3 expression and activity in primary human adipocytes

4.3.4.1 AKR1C3 expression and activity across differentiation in primary human adipocytes

AKR1C3 mRNA expression increased significantly across differentiation between preadipocytes and adipocytes in both SC and OM primary adipocytes (mean Δct for SC tissue 16.4±0.5 [preadipocytes] v 11.7±1.3 [adipocytes], p=0.03; mean Δct for OM tissue 19.5±0.1 [preadipocytes] v 11.4±1.1 [adipocytes], p=0.001, Figure 4-11). AKR1C3 activity, as determined by T generation after incubation of cells with A 200nM for 24 hours, was significantly upregulated across adipocyte differentiation in both fat depots (mean T for SC tissue 0 [preadipocytes] v 14.6±4.7nM/24h [adipocytes], p=0.02; mean T for OM tissue 0.2±0.1 [preadipocytes] v 10.9±4.9 nM/24h [adipocytes], p=0.04, Figure 4-12).

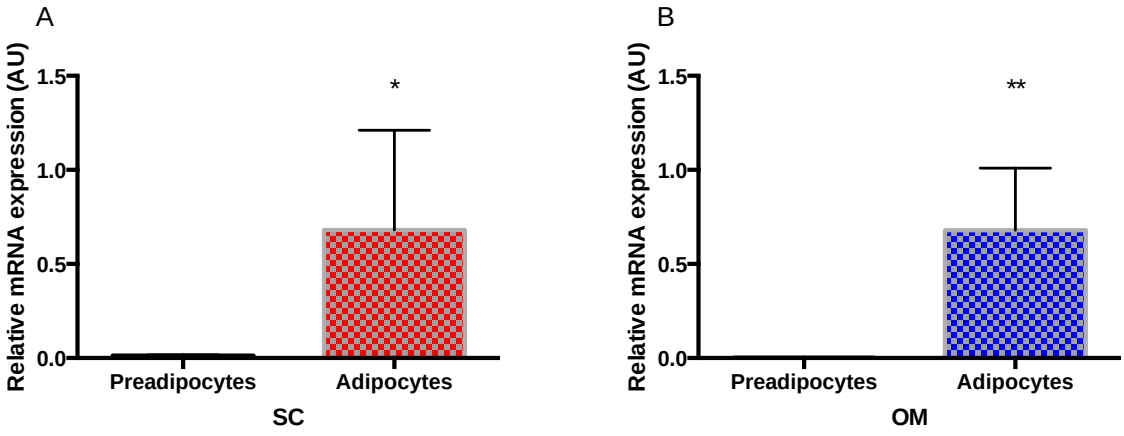


*Figure 4-11 Relative AKR1C3 mRNA expression across differentiation in primary human (A) SC and (B) OM adipocytes. Data presented as mean±SEM ⊿ct of n=3 experiments. *p<0.05; **p<0.01 compared to preadipocytes.*

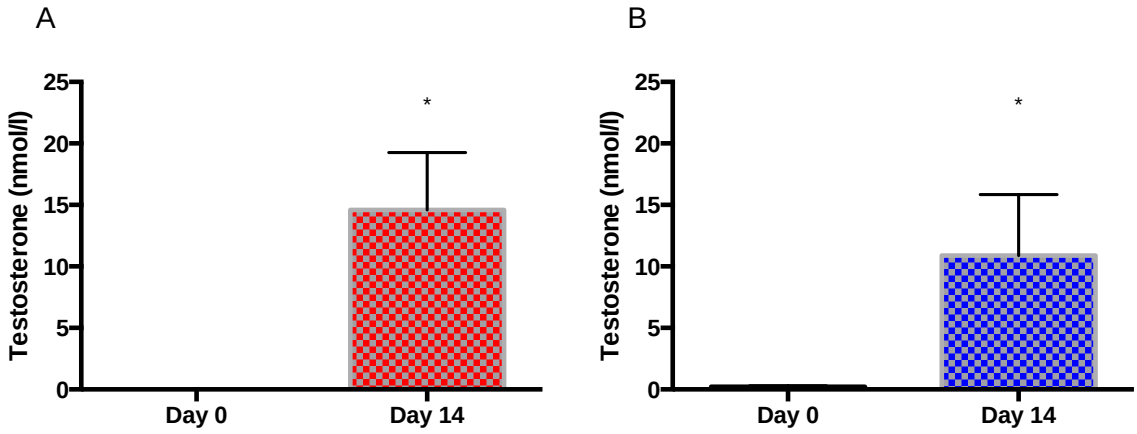


*Figure 4-12 AKR1C3 activity as determined by testosterone generation from androstenedione (200nM) over 24 hours in primary SC (A) and OM (B) adipocytes across adipocyte differentiation. Data presented as mean±SEM ⊿ct of n=3 experiments. *p<0.05 compared to Day 0.*

4.3.4.2 Effect of insulin on AKR1C3 expression and activity in primary human adipocytes

AKR1C3 mRNA expression increased significantly in primary differentiated SC adipocytes from female patients (n=3) after incubation with insulin at a dose of 20nM compared to control (mean Δct 10.1±0.9 [control] v 7.6±0.2 [insulin 20nM], p=0.049, Figure 4-13). No effects on AKR1C3 expression were observed in SC adipocytes at a dose of 10nM insulin. Similarly, incubation with insulin did not significantly increase AKR1C3 mRNA expression in differentiated OM adipocytes from females (n=3, mean Δct 9.8±0.2 [control] v 10.9±0.7 [20nM insulin], p=0.18, Figure 4-13).

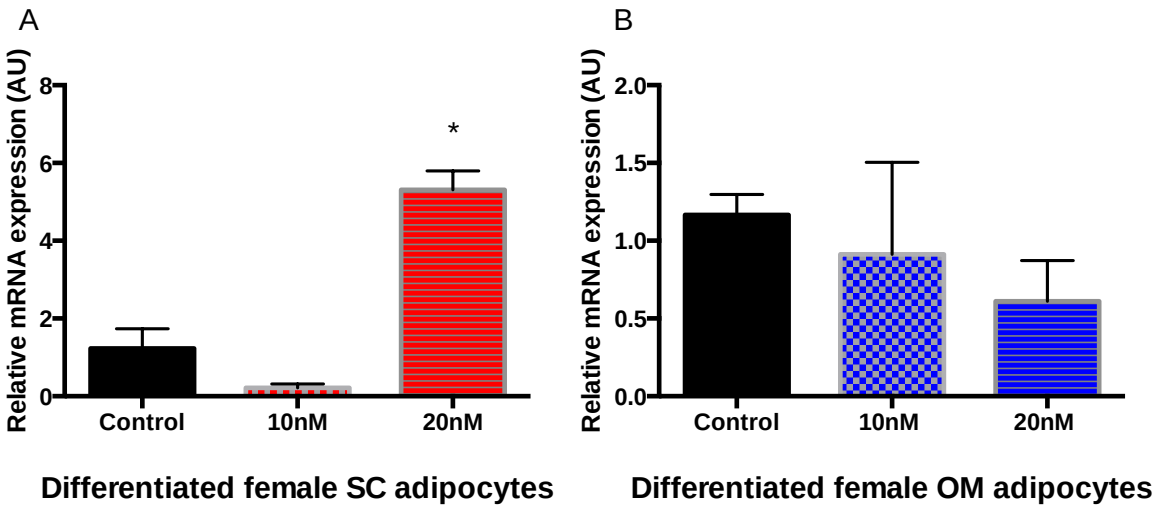


*Figure 4-13 Effect of insulin on AKR1C3 mRNA expression in (A) primary female SC adipocytes and (B) primary female OM adipocytes. *p<0.05 compared to control.*

In primary differentiated SC adipocytes, incubation with insulin 10nM significantly increased AKR1C3 activity compared to control (100% [control] v 118.3±3.1% [10nM], p=0.004, Figure 4-14). Activity in cells treated with 20nM insulin was not significantly increased (p=0.44). Insulin did not significantly increase AKR1C3 activity in primary differentiated OM adipocytes compared to control, although a trend was observed at a dose of 20nM (100% [control] v 93.1±6.8% [10nM], p=0.27; 100% [control] v 148.4±27.1% [20nM], p=0.14).

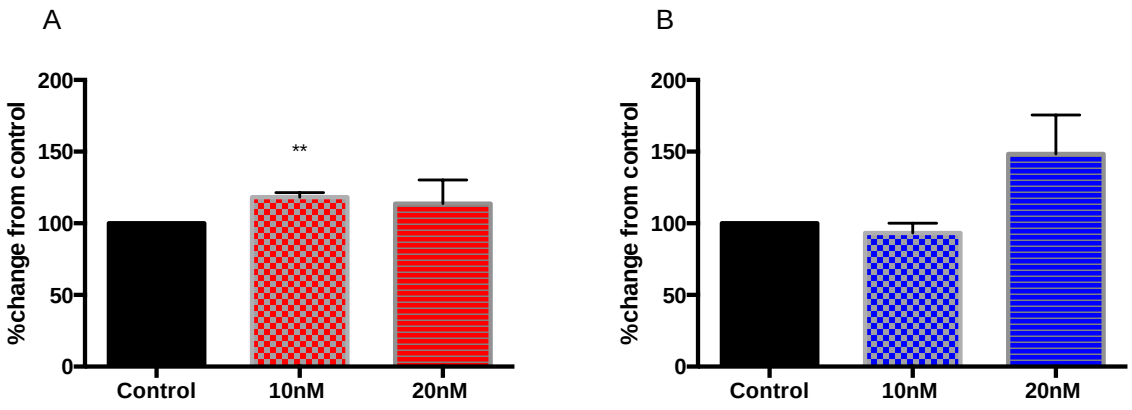


*Figure 4-14 Effect of insulin compared to control on AKR1C3 activity in primary SC (A) and OM (B) adipocytes. **p<0.01 compared to control.*

4.4 Discussion

The observations in this chapter highlight a key role for adipose tissue as an organ of androgen generation in human health and disease. Tantalisingly, these data suggest that hyperinsulinaemia and obesity, both highly prevalent in PCOS, may be potent regulators of adipose androgen metabolism, and appear to preferentially drive androgen activation in SC compared to OM adipose tissue. Our findings of a greater relative contribution by SC compared to OM fat to adipose androgen metabolism are supported by previous studies (Blouin et al., 2003, Quinkler et al., 2004, MacKenzie et al., 2008). Similarly, we found that AKR1C3 mRNA expression was highly correlated with BMI in SC tissue in females; these associations were not observed in OM fat or in males. These data broadly mirror those of Wake *et al*, who found a significant positive association between AKR1C3 expression and waist:hip ratio in SC adipose biopsies from 45 healthy men and women (Wake et al., 2007). Similar to the study by Wake, we also found obesity was associated with increased expression of the androgen-inactivating enzyme AKR1C2, but not with expression of SRD5A1. This suggests that intra-adipose conversion of A to T by AKR1C3 may be more sensitive to weight fluctuations than 5α-reduction of T to DHT by SRD5A1. Net local androgen exposure in fat is determined by the delicate equilibirium between activating and inactivating enzyme activity; increased AKR1C2 expression in obesity may therefore represent a physiological counter-balance to excessive local androgen activation by AKR1C3 (O'Reilly et al., 2014a). It is not clear which pathophysiological factors might disturb this balance in favour of increased androgen production.

Hyperinsulinaemia in PCOS is a possible complicit factor in the upregulation of adipose androgen generation. We know that insulin has a potent effect on ovarian androgen metabolism, directly driving activity of 17α-hydroxylase and other enzymes in thecal cells

(Nestler et al., 1998). AKR1C3 is a key enzyme in androgen biosynthesis in the adrenals, ovaries, prostate and adipose tissue (Nakamura et al., 2009), and polymorphisms are associated with an increased prevalence of PCOS and severity of hyperandrogenism in women (Ju et al., 2015). In the experiments described above, insulin significantly increased both expression and activity of AKR1C3 in SGBS cells. The finding of increased AKR1C3 expression with insulin was validated in primary differentiated adipocytes from the SC depot of female patients. Insulin significantly increased AKR1C3 activity in differentiated SC adipocytes, although this effect was only observed at the lower insulin dose. This observation may be due to insufficient replicate experiments, the notorious variability of primary culture and a lack of sufficient samples from male and female patients to power analysis of sex-specific effects. No significant increases in AKR1C3 activity after insulin exposure in primary OM adipocytes were observed, mirroring our mRNA expression data.

There is an emerging body of observational evidence to support a role for insulin in adipose androgen generation. Kruppel-like factor 15 (KLF15), which acts as transcriptional regulator of human AKR1C3 (HSD17B5) by binding to its promoter region, is potently stimulated by insulin in human adipose tissue (Mori et al., 2005), and appears to mediate both T generation and adipogenesis in response to insulin (Du et al., 2009). KLF15 plays a role in glucose homeostasis, and is highly expressed in liver, fat, muscle, heart and other metabolic tissues (Gray et al., 2002); targeted hepatic deletion in mice leads to reduced hepatic gluconeogenesis and hypoglycaemia (Gray et al., 2007). A further possible mechanistic link between insulin and adipose AKR1C3 regulation could be the transcription factor CAAT/Enhancer-binding protein-β (C/EBP-β), which has recently been identified as essential for transcription of HSD17B8 in the liver (Villar et al., 2007); C/EBP-β activity is also stimulated by insulin (Guo et al., 2001). In steroidogenic tissues such as fat,

hyperinsulinaemia, leading to increased expression of KLF15, C/EBPβ and other important transcription factors, could directly drive adipose AKR1C3 activity, providing a link between metabolic dysfunction, adipose tissue and androgen activation.

It is interesting that incubation of insulin-stimulated adipocytes with wortmannin did not significantly reduce AKR1C3 activity as determined by T generation. These data would suggest that insulin-induced effects on adipose androgen metabolism are not mediated via the PI3K pathway downstream from the INSR. Furthermore, previous studies have shown that insulin resistance in PCOS does not appear to encompass ovarian steroidogenic responses to insulin, with granulosa and thecal cells from PCOS ovaries demonstrating normal physiological responses to insulin stimulation in culture (Willis et al., 1996, Poretsky, 1991). This raises a number of distinct possibilities. Firstly, insulin could exert its effects on steroidogenesis independent of the INSR, perhaps via the type I or type II IGF receptors. Insulin is known to upregulate the IGF-I receptor, and amplifies the actions of IGF-I and IGF-II at the level of the ovary (Poretsky et al., 1999). IGFs are important regulators of human follicle growth, and accelerated preantral follicle growth in PCOS may be due to increased endogenous IGF action, or due to the action of insulin on the type I or type II IGF receptors (Stubbs et al., 2013). However, other studies have suggested that this is unlikely to be the case, and that the ovary maintains selective sensitivity to insulin action via the INSR in PCOS (Willis and Franks, 1995). Whether these data can be extrapolated to adipose tissue is less clear; adipose insulin resistance has been well-characterised in PCOS (Ciaraldi et al., 1998, Dunaif, 1997). A more plausible possibility is that insulin action on steroidogenesis in adipose tissue, the ovary and other organs is mediated via the mitogenic MAPK pathway after INSR binding and not via the metabolic PI3K pathway. We know that MAPK activation mediates most of the growth-promoting effects of insulin. This possibility is supported by the inability

of wormannin to reduce T generation in SGBS cells in the experiments described above, and by a limited number of *in vitro* studies (Mendez et al., 2005, Kempna et al., 2007). Further work is needed to corroborate these findings.

Evidence for adipose-specific androgen generation in PCOS supports the theory that weight loss may be a potent strategy to ameliorate the clinical and biochemical phenotype in obese women with the disorder. We found a strong positive correlation between SC adipose AKR1C3 expression and BMI in female patients in the study described above. AKR1C3 expression was significantly downregulated in 6 women after a weight loss intervention in the study by Quinkler *et al* (Quinkler et al., 2004). In a recent cohort study, serum testosterone levels in postmenopausal women fell by approximately 10% for each kg/m^2 reduction in BMI (Jones et al., 2013). Another study showed that weight loss is an effective strategy to lower both serum androgens and oestrogens in postmenopausal women, and may thereby afford protection against development of breast cancer (Campbell et al., 2012). A recent weight loss intervention using metformin with or without a novel phosphodiesterase inhibitor in 31 women with PCOS found significant reductions in weight in both groups, mirrored by reductions in serum androgens (Jensterle et al., 2014). Bariatric surgery is also likely to be an effective intervention in the hyperandrogenic morbidly obese. Two studies showed normalization of serum androgens, including DHEAS, after bariatric intervention in obese women with PCOS (Escobar-Morreale et al., 2005, Kopp et al., 2006). Another study showed that bariatric surgery significantly reduced serum androgen levels in obese women without PCOS and that this effect was observed even when preoperative levels were within the normal reference range (Ernst et al., 2013). Although some of the observed effects of weight loss on androgen metabolism may be attributable to increases in SHBG in the context of increased insulin sensitivity, changes in A and free T cannot be explained by increases in binding

proteins alone. Weight loss may also improve insulin sensitivity, thereby reducing the effects of hyperinsulinaemia on adipose androgen activation. Clinicians should have a low threshold to offer bariatric intervention to severely obese patients with androgen excess and subfertility.

Selective inhibition of AKR1C3 in adipose tissue may offer a novel therapeutic strategy to reduce local androgen exposure in PCOS. We significantly reduced adipose testosterone generation in the above experiments with a highly selective AKR1C3 inhibitor, even under insulin-stimulated conditions. AKR1C3 inhibition is currently under investigation as a possible future therapeutic direction in castration-resistant prostate cancer (Liedtke et al., 2013). It is worth remarking that AKR1C3 is also over-expressed in invasive hormone-positive ductal carcinoma of the breast (Shibuya et al., 2008), which might be importance in the context of developing future anti-proliferative treatments. Non-steroidal anti-inflammatory drugs (NSAIDs) such as salicylic acid have inhibitory effects on various AKR1C isoforms; some, such as indomethacin, have selective activity against AKR1C3 (Bauman et al., 2005), and might have a useful role in future clinical studies in PCOS. Modulation of androgen activation selectively in adipocytes could offer a novel therapeutic option in PCOS by ameliorating the adverse consequences of intra-adipose androgen generation. The systemic consequences of androgen excess in women have been investigated and discussed extensively. However, it is not clear how adipose androgen generation might impact locally on adipocyte biology, including adipogenesis and lipid metabolism, and this will be explored in chapter 5.

5 Androgen action on adipose lipid metabolism and pancreatic insulin secretion *in vitro*

5.1 Introduction

In chapter 4, we demonstrated that human adipocytes are capable of androgen activation and secretion. In obesity and under hyperinsulinaemic conditions, both highly prevalent in PCOS, this process of adipose androgen generation appears to be upregulated. It is now increasingly clear that androgens exert important effects on adipose tissue and are capable of regulating adipocyte function (O'Reilly et al., 2014a). It therefore seems reasonable to hypothesise that androgens generated in fat via this intracrine pathway are capable of modulating local adipocyte biology, including regulation of adipose tissue mass. The key processes involved in regulation of adipose tissue mass are hyperplasia (increase in fat cell number) and hypertrophy (increase in fat cell size) (Hausman et al., 2001). Hyperplasia is driven by preadipocyte proliferation and differentiation into adipocytes (adipogenesis). Hyperplasia depends upon a number of crucial transcriptional regulators and hormonal signals and has been discussed in section 1.6.3.1. Differentiation into mature adipocytes is synonymous with induction of genes involved in lipid metabolism. Upon maturation, adipocyte size is then determined by lipid flux, which refers to the tightly regulated balance between lipid accumulation and lipid breakdown (Gathercole et al., 2011); *de novo* lipogenesis, glucose uptake and FFA uptake drive lipid accumulation (and hence adipocyte hypertrophy), while lipolysis and β-oxidation favour lipid breakdown. Data on the role of androgens in regulation of lipid flux and adipocyte size are thus far conflicting.

Lipogenesis refers to the *de novo* synthesis of fatty acids within the adipocyte, uptake of dietary FFAs into the adipocyte, and re-esterification of these FFAs with glycerol to form TAG which can be stored as fuel; *de novo* lipogenesis may account for up to 20% of lipid turnover within adipose tissue (Strawford et al., 2004). As discussed in section 1.6.4, the rate-limiting step in this process is the carboxylation of mitochondrial-derived acetyl CoA to

malonyl CoA in an ATP-dependent reaction by ACC1; malonyl CoA is subsequently converted by a multi-step reaction to palmitate by FAS (Ruderman et al., 2003). ACC2 is localized to the mitochondrial membrane, where it serves as a major negative regulator of β-oxidation by generating malonyl CoA and thereby inhibiting CTP1. ACC1 and ACC2 are themselves negatively regulated by AMPK-induced serine phosphorylation, which promotes β-oxidation and lipid mobilization in low energy states. In the fed state, insulin then inactivates AMPK, allowing ACC1 and ACC2 to predominate and drive lipid accumulation.

It has been shown that glucocorticoids drive lipogenesis by upregulation of ACC expression and activity in the fed state (Gathercole et al., 2011). However, studies on the role of androgens in this and other key processes in lipid metabolism are lacking. There is ample observational data to suggest that androgens play an important role in fat mass expansion and distribution in females. Hyperandrogenic women with PCOS have higher global adiposity and visceral adipose tissue compared to control women. Intra-abdominal adiposity correlates with serum androgens (Borruel et al., 2013), and circulating androgens in women are positively associated with BMI (Mongraw-Chaffin et al., 2015). *Ex vivo*, prenatally androgenized female mice have increased adipocyte size compared to control animals (Roland et al., 2010). However, to our knowledge, there are no comprehensive studies on the effect of androgens on *de novo* lipogenesis, FFA uptake, β-oxidation and glucose uptake under fasted or fed conditions. Furthermore, insulin is a critical hormonal regulator of adipocyte lipid metabolism but there are few data on the effect of androgens on pancreatic β-cell function and insulin secretion. Limited data suggest that androgens may drive insulin secretion through the androgen receptor in the female sheep pancreas (Rae et al., 2013), and *in vivo* markers of insulin secretion may be increased in PCOS women compared to controls (Panidis et al.,

2006). Androgen action on β-cell insulin secretion could have significant consequences for lipid metabolism, with secondary effects on regulation of adipose tissue mass.

We hypothesise that androgens exert direct effects on adipose tissue lipid metabolism. To test this hypothesis, we will examine the effect of T and DHT *in vitro* on *de novo* lipogenesis, β-oxidation, FFA uptake and insulin-stimulated glucose uptake in SGBS cells, under both fasted and fed conditions. We also hypothesise that androgens exert effects on pancreatic β-cell function, and will characterize these effects in a cellular model.

5.2 Methods

5.2.1 Research strategy

In this chapter, we will validate the utility of SGBS cells as an appropriate cellular model for the study of lipid metabolism in humans. We will then explore the role of the potent androgens T and DHT on mRNA expression of key lipogenic genes in both SGBS cells and primary human SC adipocytes where available. We will study the functional impact of androgens on *de novo* lipogenesis in SGBS cells using isotope studies as described below. Functional studies of androgen action on β-oxidation, FFA uptake and insulin-stimulated glucose uptake in SGBS cells will also be employed to tease out the effects of androgens on these key processes of lipid metabolism.

To study the role of androgens in pancreatic β-cell physiology, we will examine mRNA expression of the AR in a mouse insulinoma cell line, and then characterize the effects of T and DHT on insulin secretion in a time- and dose-dependent fashion.

5.2.2 SGBS cell culture

Proliferating SGBS cells were seeded in 12- or 24-well plates and grown to confluence in DMEM-F12 with 10% FCS, additionally supplemented with 33μM biotin, 17μM pantothenate and penicillin-streptomycin 1% (growth media). In preadipocyte studies, cells were exposed to treatments, and biomaterial extracted, before differentiation. In adipocyte studies, SGBS cells were differentiated over 14 days in OF media, which was further supplemented according to the differentiation protocol described in section 2.2. In experiments studying the effect of androgens on mRNA expression of key lipogenic genes, androgens were added at the appropriate concentration to 0F media for the entire

differentiation period (14 days). All cells were cultured in serum-media (OF only without additives) for 24 hours before experiments.

5.2.3 Human preadipocyte cell culture

Primary human SC fat samples were obtained as described in section 4.2.2, and extracted from whole SC tissue according to the protocol described by Bujalska *et al* (Bujalska et al., 1999) and summarized in section 2.1. Briefly, adipose tissue samples were digested with type 2 collagenase at 37°C for one hour, followed by sterile filtration through layered gauze to remove solid components. After centrifugation, the stromovascular pellet containing preadipocytes was resuspended in media and plated in 12- or 24-well plates. Preadipocytes were grown to 80% confluence before differentiation into mature adipocytes over 14 days in chemically-defined media. In experiments studying the effect of androgens on mRNA expression of key lipogenic genes, androgens were added at the appropriate concentration to the chemically-defined for the entire differentiation period (14 days). All treatment protocols involved use of serum-free media without additives for 24 hours.

5.2.4 Min6 mouse insulinoma cell line

Min 6 cells were cultured to confluence as described in section 2.3. Briefly, cells were cultured in DMEM with high glucose (4.5g/l; 25nM), 10% FBS (Sigma Aldrich), 1% penicillin/streptomycin (Sigma Aldrich) and 100μM beta-mercaptoethanol. At confluence, cells were seeded into tissue culture plates and incubated at 37°C for functional studies of insulin secretion.

5.2.5 Cell treatments

All treatments were diluted as appropriate in serum-free media. In SGBS experiments, T was added at doses of 20nM and 40nM, and DHT at doses of 5nM and 20nM, to mimic physiological concentrations. In primary SC cell culture, treatments with T 20nM and DHT 5nM were used due to limited treatment well availability. In all functional studies, treatments with T and DHT were added acutely; in studies of mRNA expression of lipogenic genes, treatments were added across differentiation to mimic physiological conditions. Where relevant, the aromatase inhibitor letrozole was added at a dose of 100nM for 15 minutes before the addition of T to the cell culture media.

5.2.6 RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted using the Tri-Reagent protocol (section 2.4.1). RNA concentration and purity were assessed using NanoDrop technology as described above. One microgram of total RNA was used for reverse transcription (see section 2.4.2); cDNA was diluted 1:10 with RNase-free water. Real-time PCR was used to determine mRNA expression of the key lipogenic genes ACC1, ACC2, FAS and FABP4 in SGBS cells, ACC1 and ACC2 in primary SC adipocytes, and AR and insulin in MIN6 cells. Real-time PCR reactions were performed as described in section 2.4.3. mRNA levels were detected using an ABI 7500 sequence detection system (Applied Biosystems/Life Technologies, Paisley, UK). All probes and primers were supplied by Applied Biosystems/Life Technologies as expression assays (Taqman probe/primer mix). All reactions were normalized against two housekeeping genes, 18S and GAPDH.

5.2.7 *De novo* lipogenesis

Lipogenesis was measured by the uptake of 1-[^{14}C] acetate into the lipid component of adipocytes (Jamdar, 1978, Gathercole et al., 2011). After differentiation for 14 days, SGBS cells were washed and cultured in serum-free media for 4 hours. Cells were then treated with androgens (T 20-40nM and DHT 5-20nM) with and without insulin in serum-free media for 18 hours. The aromatase inhibitor letrozole (100nM, Sigma Aldrich) was added where appropriate 15 minutes before addition of T. Hot (1-[^{14}C]) and cold sodium acetate were then added to treatment wells for a further 6 hours. Cells were then washed three times and scraped with 250µl cold phosphate-buffered saline (PBS); cell lysate was transferred to glass TLC tubes. Folch solvent (chloroform:methanol 2:1) 5ml was added to each tube to recover the lipid fraction; samples were mixed vigorously, followed by the addition of 1ml distilled water to each tube. Samples were separated into two phases after centrifugation at 12,000 for 5 minutes – aqueous (upper) and solvent (lower), with protein interface. The solvent fraction was recovered and transferred to scintillation tubes, followed by evaporation to dryness overnight. Liquid scintillation cocktail (4ml, Fisher Scientific, Loughborough, UK) was added to each vial. Radioactivity retained in the cellular lipid was determined by scintillation counting and expressed as disintegrations per minute (DPM)/per well. Due to inter-assay variability, results were expressed as % change from controls per well.

5.2.8 β-oxidation

β-oxidation rates were measured by the conversion of [^{3}H]-palmitate to [^{3}H]-H_20 (Gathercole et al., 2011). At day 14 of differentiation, SGBS cells were cultured in low-glucose/0.5% BSA serum-free media for 6 h. Cells were then incubated with 300µl of low-glucose/0.5% BSA serum-free media with hot (1mCi/ml [^{3}H]-palmitate) and cold palmitate to

a final concentration of 100μM palmitate, and treated with (5nM) or without insulin and androgens, as described above, at 37°C. After 24 hours, incubation medium was recovered and precipitated with 600μl 10% acetic acid. The aqueous component of the mixture was recovered, and extracted with 2.5ml of 2:1 methanol:chloroform solution and 1ml of 2mM KCl:HCl per well. After addition of liquid scintillation cocktail, radioactivity was determined by scintillation counting and expressed as disintegrations per minute (DPM)/well. Due to inter-assay variability, results were expressed as % change from controls per well.

5.2.9 FFA uptake

FFA uptake was estimated by measurement of intracellular [^{3}H]-palmitate accumulation. Differentiated SGBS cells were washed and cultured in low-glucose/0.5% BSA serum free media for 6 h. Cells were then incubated with 300μl of low-glucose/0.5% BSA serum-free media with hot (1mCi/ml [^{3}H]-palmitate) and cold palmitate to a final concentration of 100μM palmitate, and treated with (5nM) or without insulin and androgens as described above. After 24 h, cells were lysed using cold PBS; lysate was recovered and radioactivity measured by scintillation counting, with results expressed as disintegrations per minute (DPM)/well. Due to inter-assay variability, results were expressed as % change from controls per well.

5.2.10 Insulin-stimulated glucose uptake

Glucose uptake was measured by analyzing the uptake of 2-deoxy-D-[^{3}H] glucose (Gathercole et al., 2007, Liu et al., 2001). SGBS cells were grown to confluence and

differentiated in 24-well plates as described above. Krebs-Ringer Bicarbonate (KRB) Buffer was prepared as follows:

- D-glucose 1.8g/L
- Magnesium Chloride (anhydrous) 0.0468g/L
- Potassium Chloride 0.34g/L
- Sodium Chloride 7.0g/L
- Sodium Phosphate Dibasic (anhydrous) 0.1g/L
- Sodium Phosphate Monobasic (anhydrous) 0.18g/L

Krebs-Ringer bicarbonate buffer was then further supplemented with 30mM HEPES (pH 7.4) and 0.5% BSA (KRBH/BSA).

SGBS cells were washed three times and incubated with 0.9ml KRBH/BSA and 2.5mM glucose at 37°C for 30 minutes. Insulin (0.5ng/ml) and treatments were then added, and the cells were incubated at 37°C for a further 15 minutes. Glucose uptake was initiated by the addition of 0.1ml KRBH/BSA buffer with 37mBq/l 2-deoxy-D-[^{3}H] glucose (GE Healthcare) and 0.1mmol/l glucose as final concentrations. Glucose uptake was terminated after 15 minutes by washing three times with cold PBS. Cell lysate was transferred to scintillation vials and 4ml liquid scintillation cocktail added. Radioactivity retained by the cell lysates was determined by scintillation counting.

5.2.11 Measuring effect of androgens on insulin secretion from MIN6 cells

MIN6 cells were seeded in 12-well plates in growth media as described in section 5.2.4. At confluence, medium was changed to serum-free, low glucose (1g/L, 5.5nM) DMEM; T (10nM and 100nM) and DHT (5nM and 50nM) were added for 24 hours (pre-treatment). After 24 hours, cells were washed and 1ml/well of fresh serum-free, low glucose

DMEM added; treatments with T and DHT at the above concentrations were again added to selected wells. A treatment time course was created by removing 50µl of media for insulin measurement at time points 0, 15, 30, 60, 120, 180, 240 and 300 minutes. Secreted insulin was measured using Mouse Insulin ELISA (Mercodia, UK) as described in section 2.6.1. Area-under-the-curve (AUC) measurements were used to compare differences between treatments across the time course.

5.2.12 Statistical analysis

Normally distributed data were analysed using unpaired student *t* tests to compare single treatments to controls; non-parametric tests were used when normality tests failed (Microsoft Excel or SPSS Version 22). When comparing means from multiple groups, one-way ANOVA with post-hoc Tukey testing was employed. All statistical analysis on realtime PCR data was performed for a minimum of n=3 on mean Δct values between different treatment groups. Due to inter-assay variability in functional studies of lipid metabolism, statistics were performed on the % change from controls per well.

5.3 Results

5.3.1 Validation of SGBS cells as a model of human adipocyte lipid metabolism

Morphological changes in SGBS cells across differentiation have already been described in 4.3.2.1 and are shown in Figure 4-3. Briefly, typical cytoskeletal changes were observed between Day 0 and Day 14, with loss of the fibroblast-like appearance of preadipocytes, and intracellular lipid accumulation consistent with differentiation into lipid-storing adipocytes. Using real-time PCR, we compared mRNA expression of key markers of lipid metabolism across differentiation, from Day 0 (preadipocyte) to Day 14 (adipocyte). Significant upregulation in mRNA expression of most key component genes in lipid metabolism was observed across differentiation as shown in Table 5-1.

Table 5-1 Changes in mRNA expression of key markers of lipid metabolism across SGBS differentiation (data expressed as mean ∆ct±SEM for n=3-5 experiments). Statistical analysis performed on ∆ct.

Gene	**SGBS Day 0 (preadipocyte)**	**SGBS Day 14 (adipocyte)**	**P-value (Day 0 v 14)**
LPL	Undetectable	6.3±0.1	<0.0001
ACC1	6.5±0.6	5.6±0.3	0.19
ACC2	9.5±0.4	5.9±0.5	0.001
FAS	5.0±0.2	4.1±0.3	0.04
FABP4	17.1±0.3	3.8±0.4	<0.0001

5.3.2 Lipogenesis

5.3.2.1 mRNA expression of lipogenic genes

Androgen exposure across differentiation significantly increased ACC1 mRNA expression compared to control cells (% change in Δct v control [100%]: T 20nM, 92.7±2.7%, p=0.02; T 40nM, 89.8±1.2%, p<0.001; DHT 5nM, 83.3±5.6%, p=0.006; DHT

20nM, 83.5±8.7, p=0.02, Figure 5-1). No other significant changes were observed in mRNA expression of key lipogenic genes after treatment with androgens.

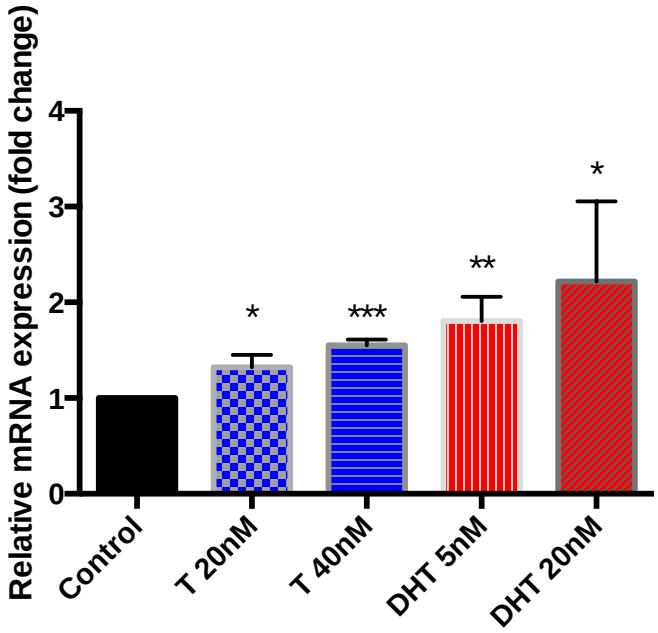


*Figure 5-1 Relative change in ACC1 mRNA expression in SGBS cells (expressed as fold change compared to control) with increasing doses of T and DHT. Statistical analysis was performed on Δct compared to control. Androgens were added to chemically-defined media throughout differentiation in order to determine chronic effects of androgen exposure. *p<0.05; **p<0.01; ***p<0.001 compared to controls.*

Table 5-2 Changes in mRNA expression in key markers of lipid metabolism after androgen exposure across differentiation in SGBS cells. Data expressed as mean ∆ct±SEM for n=3-5 experiments. Statistical analysis performed on ∆ct; p-values compared to control cells.

	Control	**T 20nM**	**T 40nM**	**DHT 5nM**	**DHT 20nM**
ACC2	5.9±0.5	6.2±0.4 (p=0.72)	6.3±0.2 (p=0.47)	6.6±0.9 (p=0.51)	6.9±1.1 (p=0.43)
FAS	4.1±0.3	4.4±0.2 (p=0.53)	4.4±0.3 (p=0.54)	4.5±1.2 (p=0.72)	4.3±1.1 (p=0.89)
FABP4	3.8±0.4	3.9±0.3 (p=0.87)	3.4±0.1 (p=0.33)	4.0±0.4 (p=0.79)	4.3±0.6 (p=0.48)

Significant changes in ACC1 mRNA expression were not observed with chronic androgen treatment across differentiation in primary SC adipocytes (mean Δct 3.1±0.9 [control] v 4.4±1.9 [T 20nM], p=0.38; v 3.5±0.5 [DHT 5nM], p=0.9). Similarly, no significant changes were observed in ACC2 mRNA expression in primary SC adipocytes after androgen exposure (mean Δct 2.1±1.5 [control] v 1.7±0.9 [T 20nM], p=0.82; v 1.1±0.5 [DHT 5nM], p=0.54).

5.3.2.2 De novo lipogenesis as measured by acetate incorporation into TAG

The effect of androgens on *de novo* lipogenesis was determined by measuring the incorporation of 1-[^{14}C]-acetate into cellular lipid. Insulin, at a dose of 5nM, significantly upregulated *de novo* lipogenesis compared to control (100% [control] v 250.0±19.1% [insulin 5nM], p=0.009, Figure 5-2).

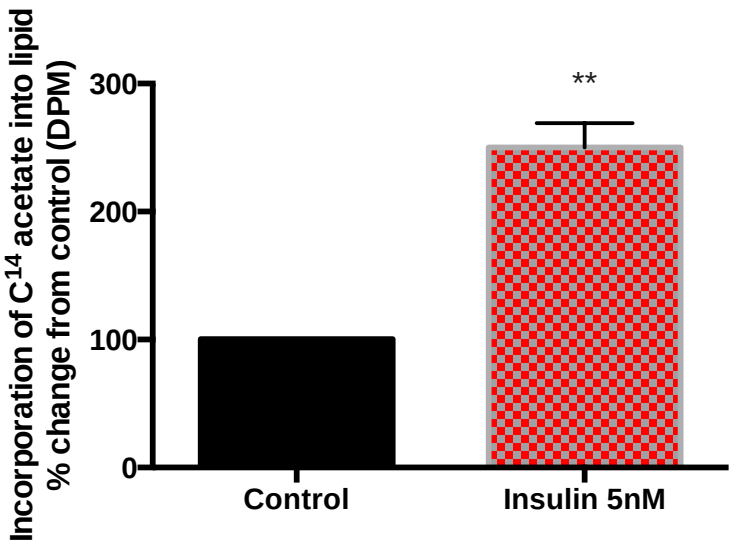


*Figure 5-2 Basal and insulin-stimulated 1-[^{14}C]-acetate incorporation into cellular lipid in differentiated SGBS adipocytes. Lipogenesis increased significantly with insulin treatment. Data are expressed as % change in disintegrations per minute (DPM) compared to control (basal, 100%). **p<0.01.*

In the absence of insulin, T 40nM increased ACC activity compared to basal controls (100% [control] v 150.4±7.3% [T 40nM], p=0.02, Figure 5-3a). This effect was also observed with DHT 50nM (100% [control] v 121.9±8.4% [DHT 50nM], p=0.03). No effects were observed at the lower androgen doses of T 20nM and DHT 10nM. Co-incubation of T-treated cells with the aromatase inhibitor (AI) letrozole (100nM) did not result in a significant change in ACC activity compared to those treated with T alone (149.5±13.2% [T 40nM+AI] v 150.4±7.3% [T 40nM], p=0.90).

In the presence of insulin, T 40nM and DHT 50nM both increased ACC activity significantly compared to basal controls (100% [control] v 260.8±82.9% [T 40nM], p=0.02; v 261.8±82.5% [DHT 50nM], p=0.04). However, neither T nor DHT in combination with insulin increased ACC activity compared to the effect of insulin alone (p=0.90 for both).

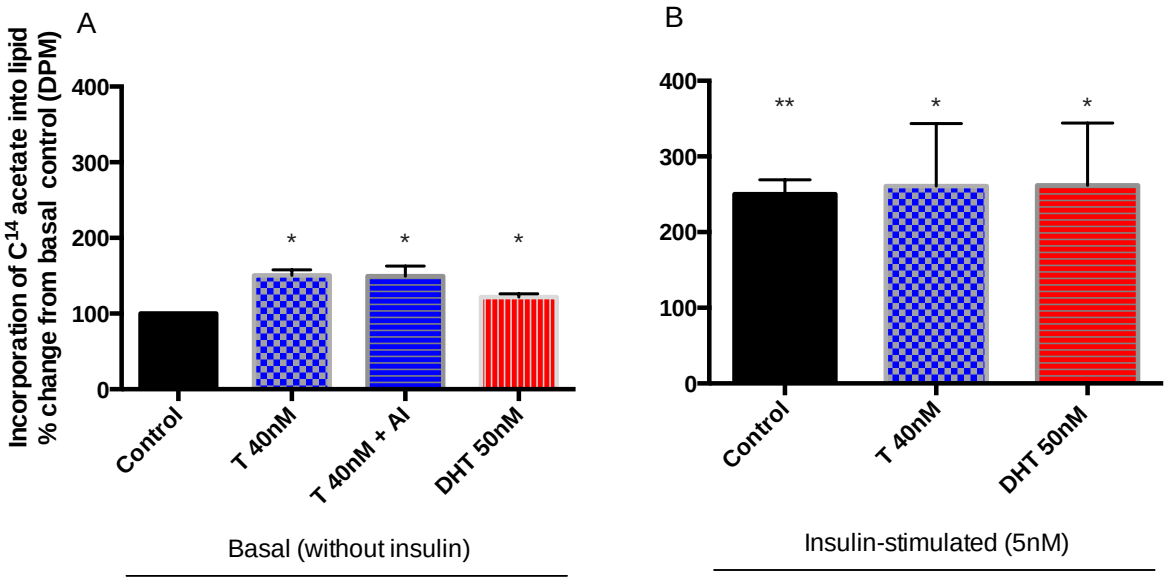


*Figure 5-3 In the absence of insulin, T 40nM and DHT 50nM significantly increased de novo lipogenesis (DNL) in differentiated SGBS cells compared to controls, as measured by incorporation of 1-[^{14}C]-acetate into cellular lipid (A). To determine if some of the observed effects of T on DNL were mediated via aromatisation of T to oestrogen, the aromatase inhibitor (AI) letrozole (100nM) was added. However, no change in the effects of T were observed. In the presence of insulin alone, DNL was significantly upregulated compared to basal controls (B). Similarly, in the presence of insulin, T 40nM and DHT 50nM significantly increased DNL compared to basal controls. However, no significant differences were observed between treatments with insulin and androgens compared to insulin alone. *p<0.05; **p<0.01 compared to basal control. Results expressed as % ±SEM change from basal control.*

5.3.3 β-oxidation

β-oxidation was measured by the conversion of [^{3}H]-palmitate to [^{3}H]-H_20 in differentiated SGBS cells. In the absence of insulin, DHT 20nM significantly reduced β-oxidation compared to basal controls (100% [control] v 83.2±5.9% [DHT 20nM], p=0.03,

Figure 5-4a). This effect was not observed with T 20nM (125.9±28.4%, p=0.4) or T 40nM (89.7±10.6%, p=0.30). Insulin failed to suppress β-oxidation in SGBS cells compared to control (100% [control] v 107.2±6.1% [insulin 5nM], p=0.28, Figure 5-4b). The addition of T 20nM, T 40nM and DHT 20nM to insulin-treated cells also failed suppress β-oxidation compared to control (100% [control] v 95.9±7.6% [T 20nM], p=0.3; v 107.5±12.4% [T 40nM], p=0.99; v 95.2±12.9% [DHT 20nM], p=0.42). Similarly, no changes in β-oxidation were observed compared to insulin alone when T 20nM, T 40nM and DHT 20nM were added to insulin treated cells (p=0.54, p=0.57 and p=0.72, respectively).

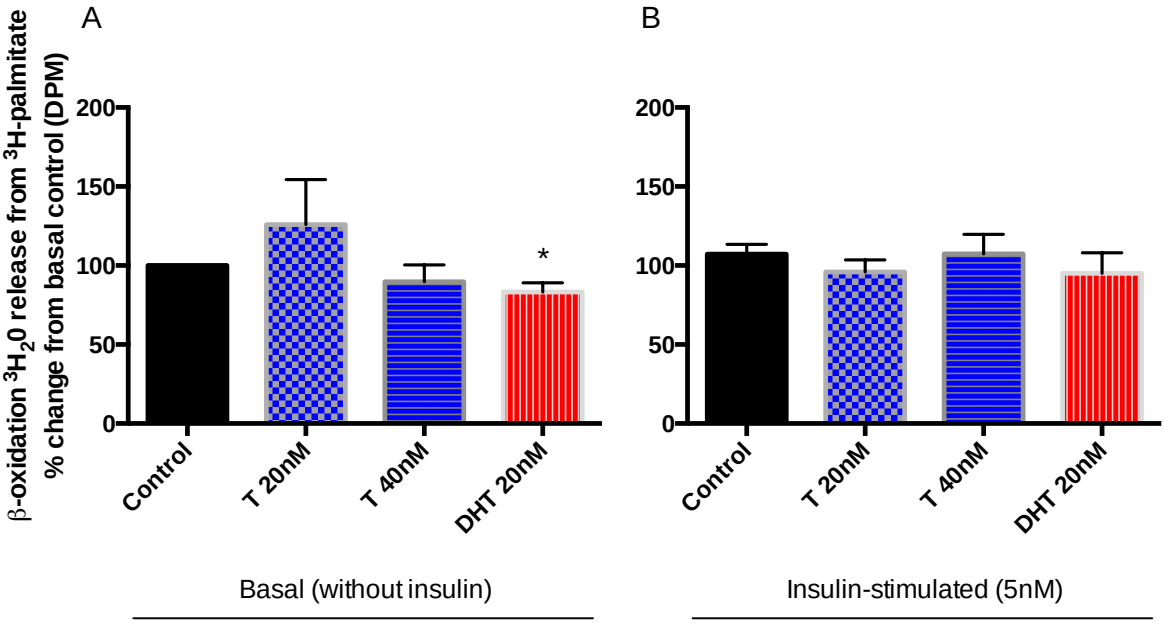


Figure 5-4 β-oxidation in SGBS cells in the absence (A) and (B) presence of insulin. Rates of β-oxidation were calculated by the conversion of [^{3}H]-palmitate to [^{3}H]-H_20. Results are expressed as % ±SEM change from basal (without insulin) controls (100%). DHT 20nM significantly suppressed β-oxidation compared to controls (A). Insulin did not suppress β-oxidation in SGBS cells. Similarly, no significant effects were observed by co-treatment with insulin and androgens (B).

5.3.4 FFA uptake

We examined the effect of androgens on FFA uptake in differentiated SGBS cells by measuring intracellcular accumulation of [^{3}H]-palmitate. Under basal conditions (absence of insulin), T 40nM did not significantly impact upon FFA uptake in SGBS cells compared to control (100% [control] v 87.1±7.9%, [T] p=0.15, Figure 5-5a). However, DHT 20nM suppressed FFA uptake (100% [control] v 86.3±6.6% [DHT 20nM], p=0.04). Insulin stimulation (5nM) significantly increased FFA uptake compared to basal controls (100% [control] v 149.7±5.4% [insulin], p=0.0001, Figure 5-5b). Co-treatment with insulin and T 40nM significantly increased FFA uptake compared to basal control (100% [control] v 165.4±28.1% [T40 nM], p=0.03), but uptake did not differ significantly compared to cells treated with insulin alone (p=0.65). Uptake did not differ significantly between basal control and those co-treated with insulin and DHT 20nM (100% [control] v 114.1±24.5% [DHT 20nM], p=0.58).

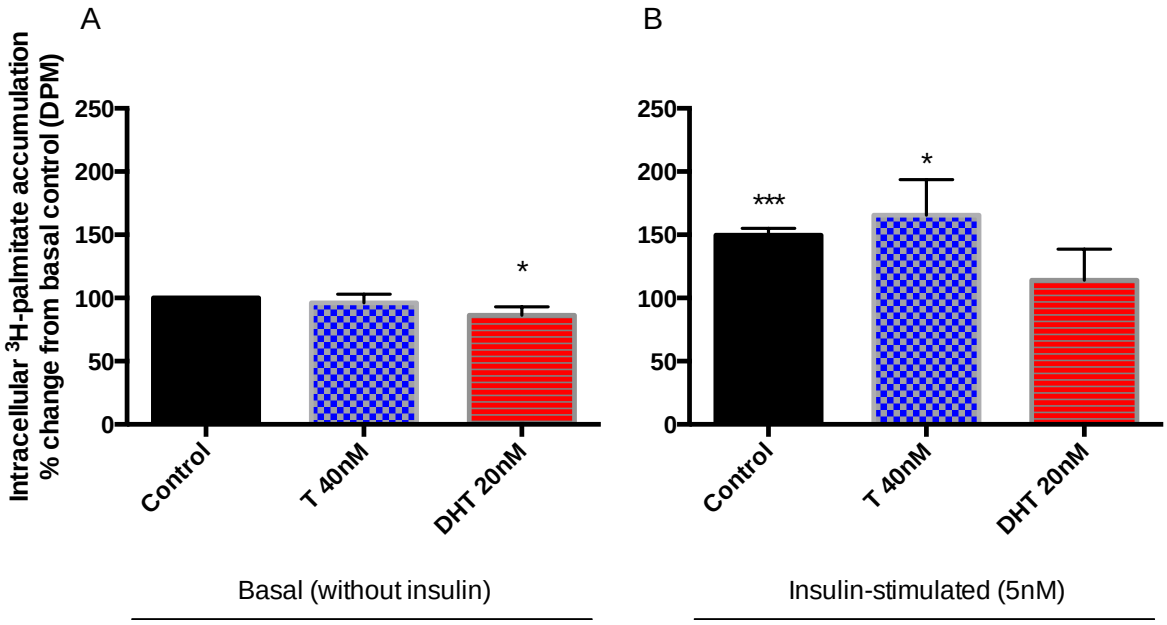


Figure 5-5 Free fatty acid (FFA) uptake in SGBS cells as measured by intracellcular accumulation of [^{3}H]-palmitate. Results are expressed as %±SEM change from basal (without insulin) controls (100%). Under basal conditions (A), DHT 20nM suppressed FFA uptake compared to controls. Both insulin and T+insulin had similar effects on FFA uptake, but co-treatment with DHT and insulin removed the stimulatory effect of insulin.

5.3.5 Insulin-stimulated glucose uptake

To assess the functional impact of androgens on insulin-stimulated glucose uptake in adipocytes, 2-deoxy-D-[^{3}H]-glucose uptake was measured in differentiated SGBS cells. Under basal conditions, T 20nM significantly increased glucose uptake compared to control (100% [control] v 113.5±4.7% [T 20nM], p=0.02, Figure 5-6). A trend towards increased uptake with T 40nM failed to reach significance (115.2±14.4%, p=0.30). Incubation with insulin alone (50nM) significantly upregulated glucose uptake compared to controls (100% [control] v 118.0±5.5% [insulin], p=0.005). Co-treatment with insulin and T 20nM did not stimulate additional glucose uptake compared to insulin alone (p=0.85); however, incubation

with insulin and T 40nM significantly increased glucose uptake compared to insulin alone (118.0±5.5% [insulin] v 164.3±19.4% [insulin + T40nM], p=0.04).

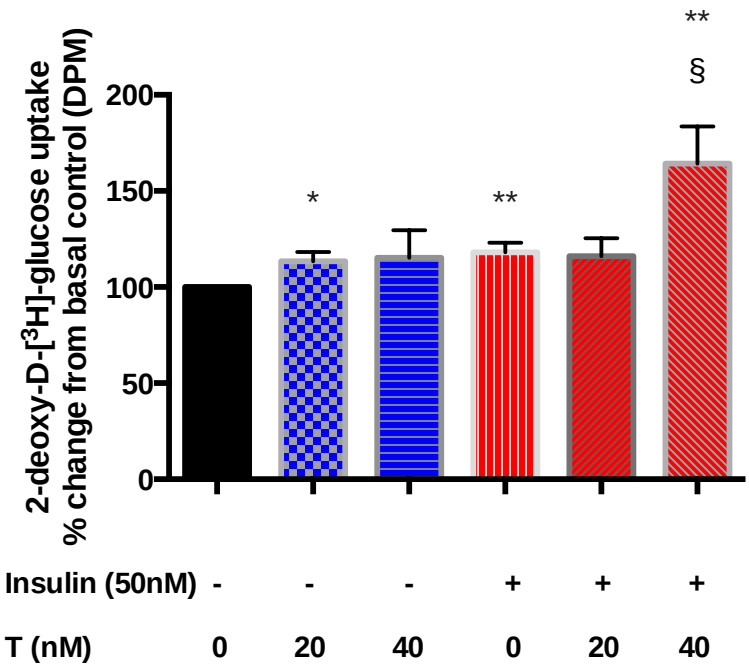


*Figure 5-6 2-deoxy-D-[^{3}H]-glucose uptake in differentiated SGBS cells (n=6). Results are expressed as % ±SEM change from basal (without insulin) controls (100%). In the absence of insulin, T 20nM significantly increased glucose uptake compared to control. Incubation with insulin significantly increased glucose uptake. Co-incubation with insulin and T also increased glucose compared to control; however co-treatment with insulin and T 40nM enhanced the stimulatory effect of insulin alone. *p<0.05; **p<0.001 compared to control; § p<0.05 compared to insulin alone.*

5.3.6 Effects of androgens on insulin secretion from MIN6 β-cell line

5.3.6.1 mRNA expression

Realtime PCR was used to measure expression of androgen receptor (AR) mRNA in MIN6 cells; mean baseline Δct values for AR were 4.5±0.1. Incubation of MIN6 with increasing doses of DHT did not significantly change either insulin (mean Δct [18S] 7.6±0.5 [control] v 7.7±0.7 [DHT 5nM], p=0.91; v 7.4±0.5 [DHT 50nM], p=0.86) or AR (mean AR Δct [GAPDH] 4.5±0.1 [control] v 5.1±0.9 [DHT 5nM], p=0.53; v 4.7±0.1 [DHT 50nM], p=0.26) mRNA expression compared to control, Figure 5-7).

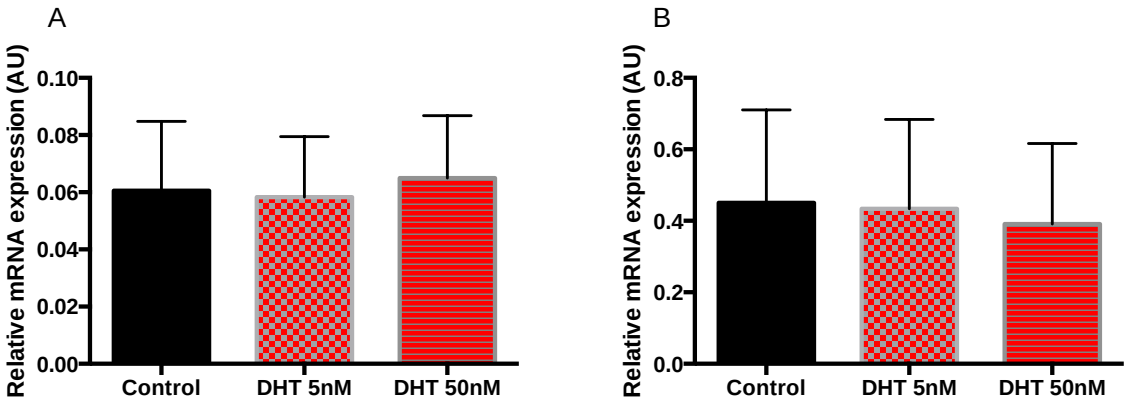


Figure 5-7 Relative mRNA expression of (A) insulin and (B) androgen receptor (AR) in the mouse insulinoma cell line MIN6. Acute treatment with DHT 5 and 50nM did not significantly change insulin or AR mRNA expression. Statistical analysis was performed on Δct values compared to control.

5.3.6.2 *Insulin secretion time course*

Insulin release from MIN6 cells in response to androgens was measured every 60 minutes for 4 hours (Figure 5-8a). Changes in area under the curve (AUC) for insulin secretion are shown in Figure 5-8b. T 10nM and 100nM increased AUC for insulin compared to control (100% [control] v 144.6±5.3% [T 10nM], p=0.02; v 340.2±144.9% [T 100nM], p=0.04). Mirroring this, significant increases in insulin secretion across the time course were observed at both concentrations of DHT (100% [control] v 573.2±31.2% [DHT 5nM], p=0.004; v 564.6±9.9% [DHT 50nM], p=0.0004).

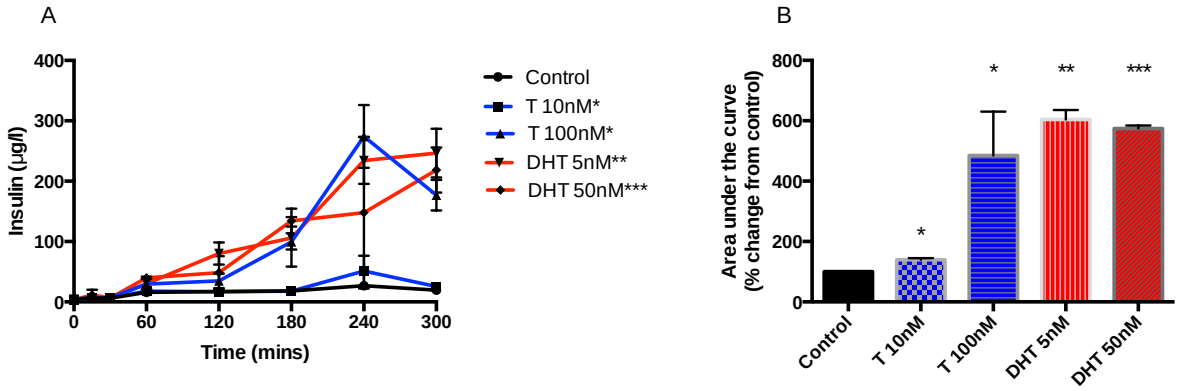


*Figure 5-8 (A) Insulin secretion time course in MIN6 pancreatic β-cell line after androgen exposure. Both T and DHT significantly increased insulin secretion compared to controls in a dose-dependent manner. (B) Area under the curve calculation for insulin shows a significant increase in secretion for both androgens at both concentrations. Results expressed as % ±SEM change in AUC compared to control (100%). *p<0.05; **p<0.01; ***p<0.001 compared to control.*

5.4 Discussion

In the above chapter, we have demonstrated important androgen-mediated effects on lipid metabolism in the SGBS cell line, which we have validated as a useful tool for the study of lipid flux in humans. The results described above suggest that androgens promote lipid accumulation in human adipocytes by driving *de novo* lipogenesis, through upregulation of ACC1, and suppressing β-oxidation, although DHT may also modestly suppress free fatty acid uptake. We could not replicate our finding of increased ACC1 mRNA expression after androgen exposure in primary SC adipocytes, but it is important to note that these experiments were restricted by a small number of replicates and limited treatment wells, and the notorious variability of primary culture should also be acknowledged. We further demonstrated that T has a stimulatory effect on glucose uptake in SGBS cells, both in the fasted and fed state, providing substrate for further lipid synthesis in the form of glycerol-3-phosphate. The net effect of these observed androgen actions on lipid flux is therefore promotion of adipocyte hypertrophy, thereby potentially fuelling the adverse metabolic phenotype observed in PCOS. We have also demonstrated that both T and DHT have potent dose-dependent insulinotropic effects in a mouse insulinoma β-cell line. Women with PCOS have relative hyperinsulinaemia compared to healthy controls (Dunaif, 1997) and although this is likely to be predominantly secondary to insulin resistance, it is reasonable to hypothesise that these differences are driven in part by androgen-mediated pancreatic insulin secretion. Androgen action as insulin secretagogues could further exacerbate metabolic dysfunction, with enhanced lipogenesis, fat accumulation and weight gain.

The observed *in vitro* associations between hyperandrogenism and lipid homeostasis support a growing body of animal data on the effects of androgens on the phenotype of WAT. In non-human primates (NHPs), androgen deprivation reduces adipocyte size in males, which

is restored by treatment with DHT (Varlamov et al., 2012). The same study found broadly similar effects of androgens on FFA uptake as we observed above, with reduced insulin-stimulated FFA uptake compared to controls in female NHP adipocytes. Androgen treatment in female rhesus macaques induces insulin resistance, not only in adult animals but also in offspring (Varlamov et al., 2013), while lipogenic gene expression is upregulated by androgens in WAT in both sexes (Varlamov et al., 2012). In rats, postnatal androgen exposure results in enlarged mesenteric adipocytes in adult animals, with insulin resistance and an atherogenic lipid profile (Alexanderson et al., 2007). These findings have been replicated in prepubertally-androgenised mice; abnormalities in DHT-treated animals include enlarged adipocyte size in the retroperitoneal, inguinal and gonadal fat depots, impaired glucose tolerance and increased atretic ovarian antral follicles (van Houten et al., 2012).

Hyperandrogenism in women is associated with central adiposity and increased waist circumference (Evans et al., 1988). Evidence suggests that androgens play a role in preferentially driving visceral fat accumulation in both animal (Nohara et al., 2014) and human (Bjorntorp, 1992) studies; recent work has also suggested that androgen exposure leads to 'masculinisation' of the proteomic signature of female adipose tissue (Montes-Nieto et al., 2013). However, as demonstrated in chapter 4, adipose tissue itself is an important organ of androgen generation and is therefore likely to contribute to circulating androgen burden in PCOS. This raises the prospect of a 'vicious circle' of adipose androgen generation and lipid accumulation, with hyperandrogenism exacerbating central fat deposition and *vice versa*. Our data suggest that androgens have important action on lipid homeostasis, with locally-generated androgens driving *de novo* lipogenesis and glucose uptake, and, in lower energy states, suppressing β-oxidation. The stimulatory effects of androgens on lipogenesis, and suppressive effects on β-oxidation, were observed under basal rather than

hyperinsulinaemic conditions, suggesting that androgens may drive lipid accumulation in the fasted state. This raises the possibility that hyperandrogenic women may be at a significant disadvantage in terms of weight loss potential from dietary interventions. These adverse effects on lipid flux and adipocyte hypertrophy may be compounded in the fed state, with increased androgen-mediated postprandial adipose glucose uptake providing further substrate for conversion to TAG, and contributing to a 'double-hit' phenomenon.

The observation of increased adipose glucose uptake after T exposure is interesting, and contrasts with that of Corbould *et al*, who demonstrated impaired insulin-stimulated glucose uptake in human female differentiated adipocytes exposed to T for 48 hours (Corbould, 2007). In our protocol, adipocytes were treated with T for 4 hours before glucose uptake was initiated, suggesting a possible divergence between acute and chronic effects of T on glucose uptake. Insulin resistance in PCOS may be tissue-specific, and one *ex vivo* study found that insulin-stimulated glucose uptake in PCOS adipocytes was comparable to that of healthy women, but was significantly reduced in skeletal myotubes (Ciaraldi et al., 2009). In the same study, whole-body insulin responsiveness of glucose uptake was significantly reduced in PCOS women. This is perhaps unsurprising, as muscle is the most critical site of postprandial insulin-mediated glucose uptake, and therefore the major determinant of whole-body insulin sensitivity. Reduced whole-body glucose uptake in the setting of androgen exposure has also been reported in other studies (Diamond et al., 1998, Polderman et al., 1994), with hepatic insulin sensitivity unchanged, supporting a role for skeletal muscle in the insulin resistance of PCOS. The elevated glucose uptake in T-treated adipocytes observed in our study could also represent a compensatory mechanism in the setting of reduced glucose uptake in skeletal muscle, but at the expense of enhanced adipose lipogenesis.

Studies of androgen action in adipose tissue are complicated by gender-specific differences in biology and should be interpreted with caution. Abdominal obesity in premenopausal women is associated with androgen excess, while in men it is linked with low circulating sex steroid levels (Mongraw-Chaffin et al., 2015). It is also interesting to note that the adverse metabolic phenotype commonly associated with male hypogonadism mirrors that of androgen excess in women (Escobar-Morreale et al., 2014), with central fat accumulation, dyslipidaemia and insulin resistance. In healthy females, high oestrogen and low androgen levels facilitate preferential accumulation of SC fat in the gluteofemoral region rather than visceral fat in the abdominal region, contributing to the so-called female 'pear-shape' (Wells, 2007). Hyperandrogenic women with PCOS are generally believed to have a more central fat distribution pattern (Escobar-Morreale et al., 2005), although some data suggest that differences reside in total fat mass rather than in a specific regional distribution (Barber et al., 2008). In healthy men, physiologically high androgen levels are associated with a degree of abdominal fat distribution, but this is also counter-balanced by the increased lean mass (largely muscle) conferred by very high T levels. In the setting of untreated male hypogonadism, with circulating androgens often broadly comparable to levels observed in PCOS, this balance is disturbed, with increased central adiposity, reduced lean mass, and an unfavourable metabolic profile (Rao et al., 2013). Further studies are required to understand these complex associations, but it is apparent that androgens play a major role in adipose lipid metabolism and regulation of fat mass in both sexes.

The effects of androgens on pancreatic β-cell insulin secretion demonstrated above were potent and are likely to be of physiological significance; AR mRNA was highly expressed in MIN6 cells. Women with androgen excess have significantly higher basal insulin secretory rates, as well as abnormal postprandial insulin release (O'Meara et al., 1993, Holte

et al., 1994). Chronic androgen exposure in female mice induces β-cell hyperfunction, and ultimately islet failure (Roland et al., 2010). β-cell function appears to be intimately linked with free T levels in PCOS. There is convincing evidence of β-cell overactivity in those with androgen excess, strongly supporting the hypothesis that hyperandrogenism drives insulin hypersecretion (Goodarzi et al., 2005). Metabolic consequences of androgen-induced stimulation of pancreatic insulin secretion include acceleration of overt β-cell failure and onset of hyperglycaemia, as well as exacerbation of adipose lipogenesis and fat mass expansion.

The data presented in this chapter support our hypothesis that androgens act at multiple levels to adversely impact on adipose lipid homeostasis, insulin action and global metabolic function. In the final results chapter, we will attempt to tease out the role of androgens in adipose tissue function and global metabolism in a dedicated *in vivo* study in both PCOS women and healthy female volunteers.

6 The role of androgens in adipose tissue biology *in vivo*

6.1 Introduction

In chapter 5, we examined the role of androgens in adipose lipid metabolism and pancreatic insulin secretion *in vitro*, demonstrating upregulation of lipogenesis in adipocytes and insulinotropic effects on β-cell function. The effects of acute androgen exposure on *in vivo* adipose physiology, lipid metabolism and insulin secretion in human subjects have not been extensively investigated. In chapter 4, we highlighted the role of adipose tissue as an intracrine androgen-generating organ, expressing androgen-activating enzymes and actively converting precursor hormones into more potent metabolites. However, the potential of adipose tissue for androgen activation after oral exposure to precursor hormones has not been explored in the human setting. Furthermore, there are few studies on the differences in intra-adipose androgen concentrations between PCOS women and the background female population. The evolution of LC-MS/MS in recent years has allowed for accurate identification and quantification of steroids in a variety of human tissues, even when present at low concentrations (Fahlbusch et al., 2015, Vitku et al., 2015). These techniques have been extended in recent years to intra-adipose steroid quantification (Methlie et al., 2013, Wang et al., 2013), but estimation of adipose-specific androgen generation has been limited to only a small number of studies to date (Wang et al., 2011).

Observational studies have shown abnormal expression of genes involved in lipid metabolism in SC abdominal adipose tissue of women with PCOS (Chazenbalk et al., 2012). Adipocyte hypertrophy appears to be more prevalent in PCOS than comparably obese control women (Manneras-Holm et al., 2011). Adipocyte diameter is closely linked to insulin resistance and metabolic dysfunction (Villa and Pratley, 2011). Our *in vitro* findings of enhanced *de novo* lipogenesis in androgen-treated adipocytes in Chapter 5 provide a plausible mechanistic link between androgen excess and adipocyte hypertrophy; further support for this

association includes the observation of T-induced suppression of lipolysis in SC adipocytes (Dicker et al., 2004). However, to our knowledge, these findings have not been tested in the *in vivo* setting after acute androgen exposure, using real-time markers of lipid flux in a human model. Equally, we are not aware of any human clinical studies examining the impact of androgen exposure on serum insulin levels. Testosterone has been shown to acutely increase insulin secretion in primary cultures pancreatic islets from rats (Morimoto et al., 2001), while androgens increased insulin secretion and β-cell number in female sheep exposed to androgens *in utero* (Rae et al., 2013). However, it is imperative to replicate our *in vitro* findings of chapter 5 in human *in vivo* studies to provide convincing evidence of their physiological significance.

We hypothesise that acute androgen exposure *in vivo* induces alterations in adipose tissue lipid homeostasis and androgen generation in women with PCOS, as well as changes in pancreatic insulin release. To test these hypotheses, we will study androgen metabolism and lipid biology after an oral DHEA challenge in PCOS women and healthy controls. This is a validated tool for the study of *in vivo* androgen physiology in human health and disease (Fassnacht et al., 2003). Androgen exposure after oral intake also provides an opportunity to study the acute consequences of hyperandrogenism on metabolic parameters and adipose tissue biology. To understand the *in vivo* associations between insulin sensitivity and androgen metabolism, we will study the hyperandrogenic phenotype of a small cohort of severely insulin-resistant women with loss-of-function mutations in the INSR (Semple et al., 2011), and measure serum insulin levels before and after DHEA in all three study groups. We will also study the consequences of androgen exposure on realtime markers of adipose lipid metabolism and insulin sensitivity, using adipose tissue microdialysis (Hazlehurst et al., 2013), as well as on adipose-specific androgen generation using LC-MS/MS.

6.2 Methods

6.2.1 Research strategy

In this chapter, we will compare patterns of serum androgen generation and urinary androgen excretion after DHEA in women with PCOS and compared to healthy volunteers. To examine the role of hyperinsulinaemia in androgen metabolism, we will measure serum androgens and urinary androgen excretion in a small cohort of women (n=3) with inactivating mutations of the INSR. Furthermore, we will test for evidence of adipose-specific androgen generation in all three groups using the technique of adipose microdialysis (Hazlehurst et al., 2013), and determine if oral DHEA intake increases local androgen activation in fat. In the baseline protocol, participants will remain fasted for the duration of the DHEA challenge test. A second study protocol will then be utilised to test the impact of caloric intake on systemic androgen metabolism, with participants receiving a standardized meal across the DHEA challenge. Patterns of fat distribution and visceral adiposity will be measured with DXA imaging and compared across the three study groups.

To study the effects of androgen exposure on adipose lipid metabolism and insulin sensitivity in realtime, levels of intra-adipose glycerol, pyruvate, lactate and glucose will be measured using microdialysis before and after oral DHEA (under fasted conditions).

6.2.2 Subjects

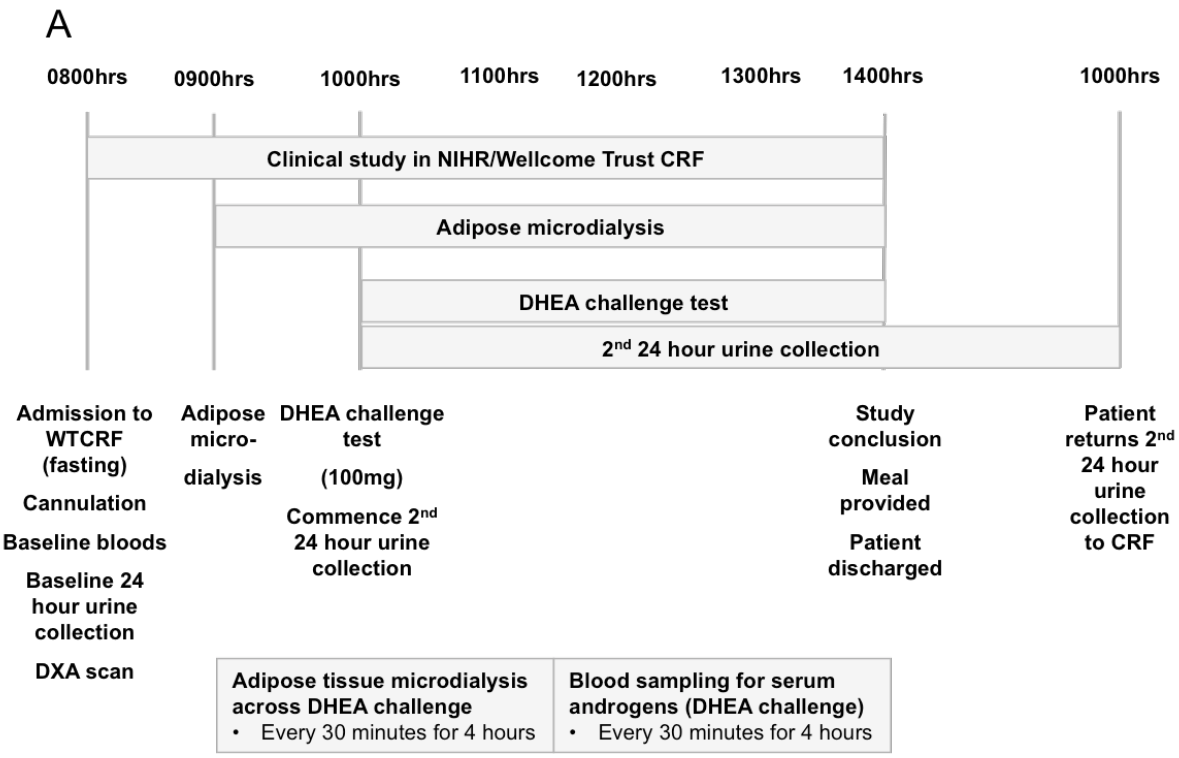
Women with PCOS were recruited from outpatient clinics at the Queen Elizabeth Hospital Birmingham (QEHB) and Birmingham Women's Hospital (BWH). This study received ethical approval from Edgbaston REC (Ref. 12/WM/0206) and all participants gave written informed consent. PCOS was diagnosed according to the Rotterdam ESHRE 2004

criteria, with the presence of two or more of the following: oligo-/anovulation, clinical signs of hyperandrogenism, and polycystic ovaries on ultrasound (Rotterdam, 2004b). Other causes of oligomenorrhoea and/or androgen excess were excluded by history, physical examination, biochemical assessment and, where appropriate, imaging. Healthy controls were recruited via local advertisement, with a diagnosis of PCOS excluded by clinical and biochemical parameters. Women with INSR mutations were recruited via the Severe Insulin Resistance (SIR) clinic at Addenbrooke's Hospital, Cambridge. Exclusion criteria for all three groups were current or recent treatment with glucocorticoids, congenital adrenal hyperplasia, hyperprolactinemia, thyroid dysfunction, pregnancy, age <18 or >45 years, oral contraceptive or oral anti-androgen therapy within 3 months prior to recruitment, and diabetes mellitus.

6.2.3 Clinical protocol

Participants attended the National Institute of Health Research/Wellcome Trust Clinical Research Facility at the Queen Elizabeth Hospital Birmingham at 08:00 after an overnight fast for a single day of integrated assessment (Figure 6-1). All patients provided a pre-collected 24-h urine sample for urinary steroid metabolite analysis by GC/MS. Baseline anthropometric assessment included height, weight and body mass index (BMI, kg/m^2) and measurement. Dual-energy x-ray absorptiometry (DXA) was employed for body composition assessment using Hologic Discovery/W DXA as described in section 3.2.3. Fat phenotype was measured by assessment of android and gynaecoid fat distribution, with subsequent calculation of android:gynaecoid fat ratio. Estimated visceral adipose tissue was calculated as volume (cm^3), mass (g) and % of total fat mass. Baseline bloods were drawn for fasting plasma glucose and insulin, as well as serum androgens and gonadotrophins.

In Protocol A (Figure 6-1A), an adipose microdialysis catheter was inserted under local anaesthetic at 09.00. At 10.00, 100mg of the oral androgen precursor DHEA (25mg capsules, Olympian) was administered. Adipose microdialysis fluid and serum were sampled every 30 minutes for 4 hours for androgen quantification by LC-MS/MS. Participants remained fasted until the study visit conclusion. Participants in Protocol A also returned a 2nd 24-hour urine sample to the Clinical Research Facility the following day so that the impact of DHEA on androgen excretion could be measured. In Protocol B (Figure 6-1B), a standardized mixed meal (carbohydrate 45g, protein 23g and fat 20g) was provided at 13.00. Adipose microdialysis was not performed in Protocol B due to the confounding effects of caloric intake on lipid metabolism. Study volunteers completed either Protocol A or Protocol B (no study crossover component), although INSR patients completed both.



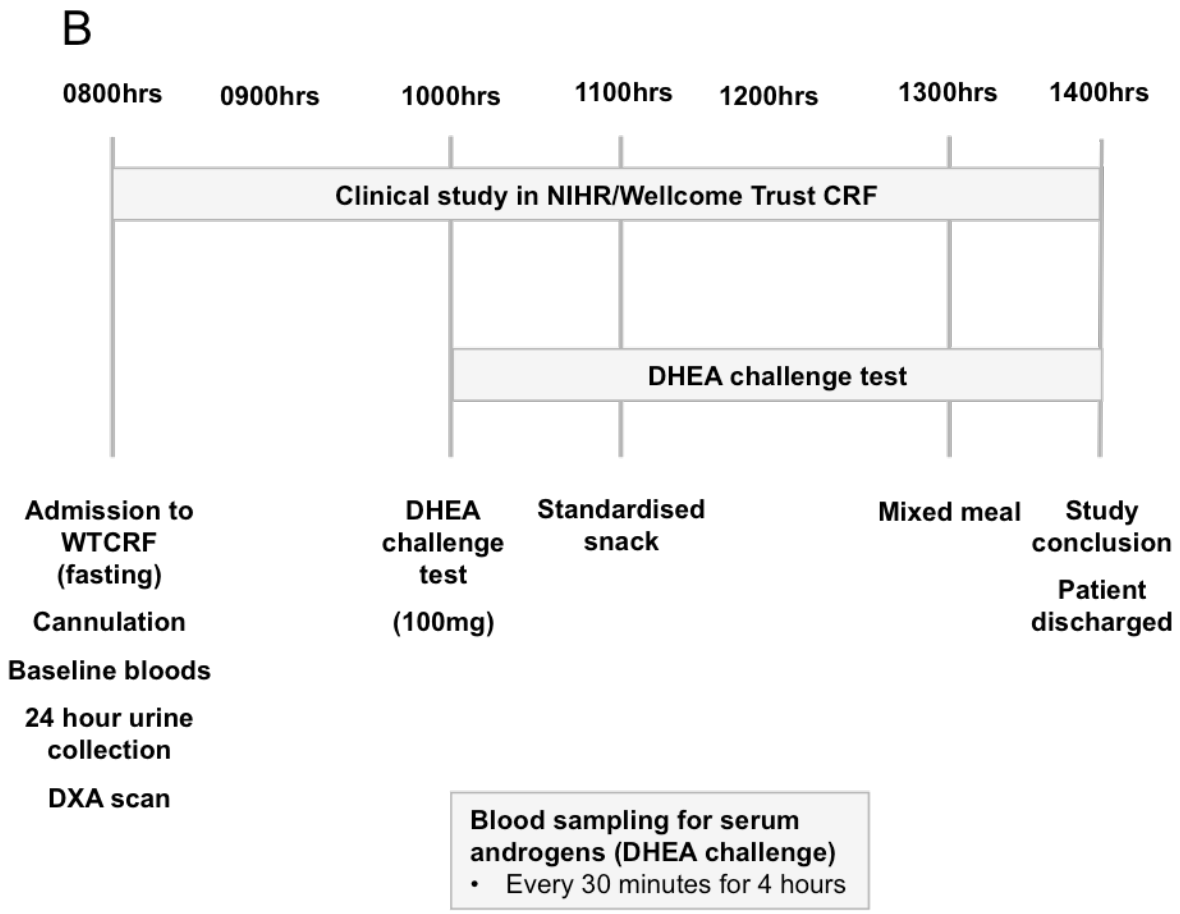


Figure 6-1 Clinical protocol. (A) Patients fasted for the duration of the DHEA challenge and adipose microdialysis. In protocol A, patients also commenced a second 24 hour urine collection at the start of the DHEA challenge test, which was returned to the WTCRF the following day. (B) In protocol B, patients were given a standardised snack at 11.00 and a mixed meal at 13.00.

6.2.4 Serum, urine and microdialysis steroid measurements

Serum androgens were measured by liquid chromatography/tandem mass spectrometry (LC-MS/MS) as described in sections 2.5.1 and 3.2.4, employing a Waters Xevo mass spectrometer with Acquity uPLC system. Briefly, LC-MS/MS conditions consisted of an electrospray ionization source with capillary voltage 4.0kV, a source temperature of 150°C, and a desolvation temperature of 500°C. Serum steroid oxime analysis was employed

for the measurement of T, A and DHEA and carried out in positive mode, whereas measurement of serum DHEAS was performed in negative mode. T, A and DHEA were extracted from serum via liquid-liquid extraction using tert-butyl-methyl-ether (MTBE), followed by derivatization into steroid oximes employing 100µl derivatization mixture. For protein precipitation and extraction of DHEAS, 20µl $ZnSO_4$ 0.1mM and 100µl acetonitrile were added to 20µl serum before evaporation under constant nitrogen flow (Chadwick et al., 2005). All steroids were separated using an optimized gradient system consisting of methanol with 0.1% formic acid and quantified referring to a linear calibration series with appropriate internal standards. Each steroid was identified by matching retention times and two mass transitions in comparison to a deuterated reference compound.

Urinary steroid metabolite excretion analysis was carried out by GC/MS as described previously in sections 2.5.3 and 3.2.4 (Arlt et al., 2004). The sum of An and Et was considered representative of active androgen metabolite excretion (Silfen et al., 2002). Net systemic 5α-reductase activity was assessed by the ratios of An/Et and 5α-THF/THF. Total glucocorticoid metabolite excretion was assessed as the sum of 5α-THF + THF + THE + cortolones + cortols + cortisol + cortisone.

For steroid extraction from microdialysis fluid, vials containing 10-15µl of fluid were defrosted at room temperature. 50µl of LC-MS/MS grade water was added to each vial and gently spun at 12,000rpm for 5 minutes. Due to small sample volumes, it was necessary to combine samples from consecutive time-points as follows: (1) Pre-DHEA: time-points -15, 0 and 30 minutes, (2) Post-DHEA (A): time points 60, 90, 120 and 150 minutes and (3) Post-DHEA (B): time-points 180, 210 and 240 minutes, giving volumes of 150, 200 and 150µl respectively per combined sample in each patient. Samples were then added to sylinised tubes and reconstituted with water to give a final volume of 500µl. A calibration series was

prepared using serum steroid calibrants C0-C11 as described above. Internal standard 20µl was then added to each sample. MTBE 2.5ml was added to each sample, followed by vortexing and freezing for at least 60 minutes at -20°C. MTBE was then poured into next sylinised tubes and evaporated to dryness under nitrogen at 55°C, followed by reconstitution with 125µl methanol/water (50:50) and transfer to 96-well plates. Plates were stored at -20°C until LC-MS/MS analysis.

6.2.5 Adipose microdialysis

Adipose microdialysis was carried out as described by Tomlinson, Figure 6-2 (Tomlinson et al., 2007). A microdialysis catheter (CMA Microdialysis, Stockhold, Sweden) was inserted into the SC abdominal fat, approximately 10cm lateral to the umbilicus, under local anaesthetic and sterile technique. After an initial 5-10 minute flush sequence (15µl/min), microdialysis proceeded at a rate of 0.5µl/min; samples were collected at -15 and 0 minutes pre-DHEA administration, and subsequently every 30 minutes for 4 hours. Samples were analysed for metabolic markers of insulin action and lipid metabolism, and for adipose-specific androgen levels.

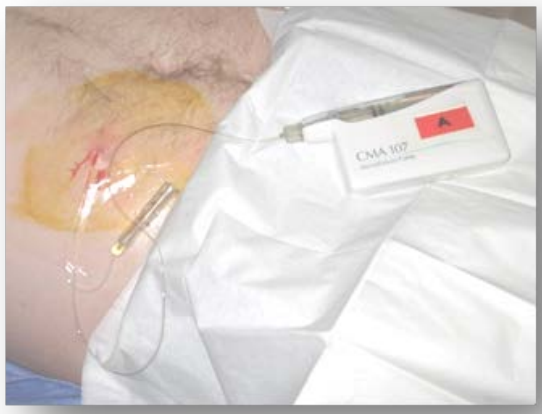


Figure 6-2 Adipose microdialysis in SC abdominal adipose tissue (CMA Microdialysis, Sweden) (Tomlinson et al., 2007).

6.2.6 Serum insulin and FFAs

Serum insulin was measured using a Human Insulin Elisa kit (Mercodia, Sweden) as described in section 2.6.1. Briefly, calibrators and samples (10µl each) in duplicate were transferred to a 96-well plate. Enzyme conjugate 1X solution was added to each well, and samples incubated on a plate shaker (700-900 rpm) for 2 hours at room temperature. The plate was then washed manually by adding 350µl wash buffer 1X solution to each well, followed by addition of Substrate TMB to each well. After incubation for 15 minutes at room temperature, Stop Solution was added and the plate placed on the shaker for 5 seconds to ensure mixing. Optical density of plates was measured at 450nm on spectrophotometer. Serum FFAs were measured as described in section 2.6.2, using a commercially available kit (ZenBio, NC, US).

6.2.7 Statistical analysis

Statistical Package for the Social Sciences (SPSS, Chicago, Il.) Version 21 was used for data analysis. All data is expressed as mean ± standard deviation (SD) unless otherwise stated. Data was log-transformed if necessary to normalize before using parametric tests. Independent samples *t* tests or Mann-Whitney were used as appropriate for comparison between two groups. One-way ANOVA with *post hoc* Tukey testing was used for multiple comparisons between different groups. Area-under-the-curve (AUC) across the DHEA challenge test was measured using the Trapezoidal Rule (Microsoft Excel). Categorical variables were compared using Pearson's Chi-Square test. Correlation testing was performed on continuous variables using Pearson's correlation co-efficient for normally distributed data or Spearman's test for non-normally distributed data. Differences were considered statistically significant at $p<0.05$.

Consultation with a dedicated university statistician was necessary in order to obtain accurate power calculations for this clinical study. Based on estimated detectable differences and standard deviations in adipose microdialysis values, serum steroid levels and urinary metabolite excretion profiles, a sample size of 10 PCOS and 10 control patients was deemed sufficient to achieve 80% statistical power. This statistical powering could not be applied to the INSR cohort due to the small number of available study patients.

6.3 Results

6.3.1 Serum androgens and urinary steroid excretion

6.3.1.1 Baseline comparison

A total of 8 women with PCOS, 8 healthy controls and 2 INSR women completed Protocol A (fasted conditions). A further 10 PCOS, 10 healthy controls and 3 INSR women (two of whom also participated in Protocol A) took part in Protocol B (fed conditions). Patients attending for Protocols A and B were matched for age and BMI. Baseline characteristics, baseline serum androgens and urinary steroid excretion of all study participants (Protocols A and B combined) are shown in Table 6-1.

All three participant groups were matched for age and BMI. Women with PCOS had higher serum levels of T, A and DHEA than healthy controls. Trends were observed towards higher urinary An/Et and 5α-THF/THF ratios (markers of 5α-reductase activity), and higher total urinary androgen and glucocorticoid metabolite excretion, in PCOS women compared to the other two groups, but these did not reach significance. HOMA-IR was significantly higher in INSR patients ($p<0.0001$); serum triglycerides were higher in PCOS women compared to controls ($p=0.01$).

Table 6-1 Baseline characteristics, serum androgens and urinary steroid profiles of healthy controls, PCOS and women with INSR mutations (both study protocols combined). Data expressed as mean±SD unless otherwise stated.

a, p<0.05, b, p<0.01, c, p<0.001 as compared to BMI-matched healthy controls. d, p<0.05; e, p<0.01; f, <0.001 as compared to PCOS.

Variable	**Healthy controls (n=18)**	**PCOS (n=18)**	**INSR mutations (n=3)**
Age (years)	29.9±5.8	29.9±6.2	25.7±7.6
BMI (kg/m^2)	27.2±5.1	31.9±7.5	29.1±2.6
HOMA-IR	1.3±1.3	4.8±4.4	44.9±41.5[c, f]
Total cholesterol (mmol/l)	3.7±1.2	4.3±1.3	5.3±0.9
HDL cholesterol (mmol/l)	1.5±0.3	1.5±0.6	2.1±0.5
Triglcyerides (mmol/l)	0.6±0.2	1.3±0.9[a]	1.0±0.4
AST (U/L)	16.2±3.2	25.5±18.9	13.7±2.0
Testosterone (nmol/l)	0.2±0.4	0.7±0.8[a]	0.1±0.2
Androstenedione (nmol/l)	3.9±3.2	6.9±3.8[a]	4.6±2.4
DHEA (nmol/l)	10.1±10.1	22.8±13.4[b]	14.3±11.6
DHEAS (µmol/l)	6.4±5.1	7.0±4.1	4.1±3.2
FAI (T*100/SHBG)	2.3±1.5	4.3±2.4	5.2±6.9
An/Et ratio (5α-reductase activity)	1.1±0.3	1.4±0.6	1.2±0.6
5α-THF/THF (5α-reductase activity)	0.8±0.3	1.1±0.6	1.0±0.7
Total androgen metabolites (An+Et)	3072±2447	4093±3623	3246±2162
Total glucocorticoid metabolites	4864±2757	6098±3535	3789±667

6.3.1.2 DHEA challenge test and serum androgens

Serum androgens across the DHEA challenge test for Protocol A and B are shown in Figure 6-3 and Figure 6-4 respectively. Baseline T levels did not differ significantly between PCOS women participating in the fasted and fed studies (p=0.70) or between the two corresponding healthy control groups (p=0.81). However, baseline A levels were higher in the PCOS women in Protocol B (fed) compared to those from Protocol A (9.3±3.3 v 3.8±1.6nmol/l, p=0.001). The same observation was noted in healthy controls, with higher baseline A levels in the Protocol B (fed) group compared to those from Protocol A (5.6±3.4 v 1.7±1.1, p=0.03). Baseline DHEA and DHEAS levels did not differ significantly between the two PCOS groups (p=0.41 and p=0.99, respectively) or the two control groups (p=0.95 and p=0.98, respectively).

There was a trend towards higher AUC (mean±SEM nmol/l*min) values in PCOS compared to controls for T (156±43 [PCOS] v 78±27 [controls], p=0.15) and A (1065±245 [PCOS] v 683±49, p=0.15) in the fasted protocol, but these did not reach statistical significance. AUC for T in INSR patients in the fasted protocol was significantly higher than in both PCOS and controls (731±46 [INSR] v 156±43 [PCOS], p<0.0001; v 78±27 [controls], p<0.0001). AUC for A was also higher in fasted INSR patients compared to PCOS and controls (4633±197 [INSR] v 1065±245 [PCOS], p<0.0001; v 683±49 [controls], p<0.0001).

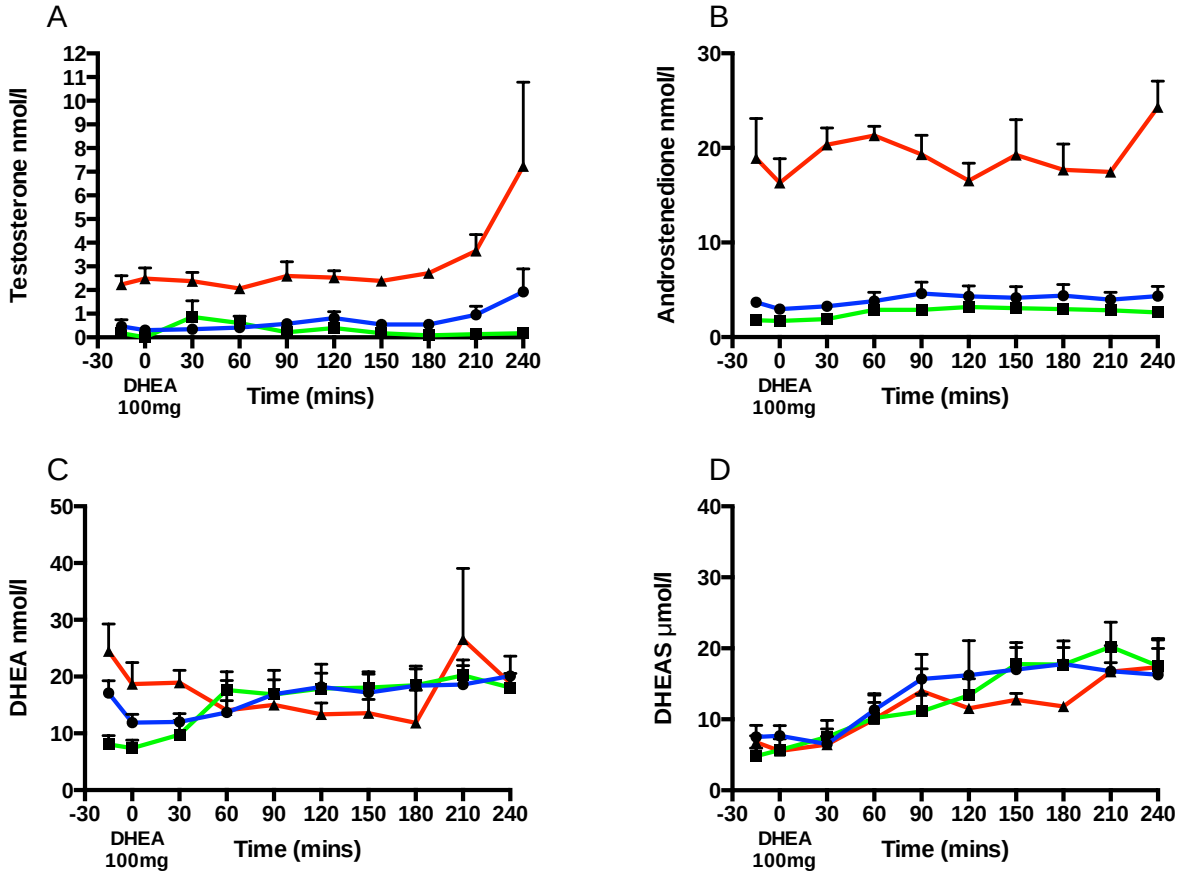


Figure 6-3 Serum testosterone (A), androstenedione (B), DHEA (C) and DHEAS (D) across the DHEA challenge in Protocol A (fasted) in healthy controls (green line), PCOS (blue line) and INSR mutation women (red line).

In the fed state (Figure 6-4), AUC (mean±SEM nmol/l*min) for T and A was significantly higher in PCOS women compared to controls (258±65 v 63.3±25.1, p=0.01, and 2507±237 v 1556±303, p=0.01, respectively). AUC for T did not differ significantly between INSR patients and controls (226±213 v 63±25, p=0.17); however AUC for A was significantly higher in INSR patients than in controls (2533±209 v 1556±303, p=0.02). AUC values for A and T did not differ significantly between INSR and PCOS (p=0.84 and p=0.96, respectively).

AUC values (mean±SEM nmol/l*min) for T were similar across fed and fasted conditions in both PCOS women (p=0.23) and healthy controls (p=0.67); however AUC values for A were significantly higher in women participating in the fed protocol compared to their fasted counterparts, in both PCOS women (p=0.0007) and healthy controls (p=0.008).

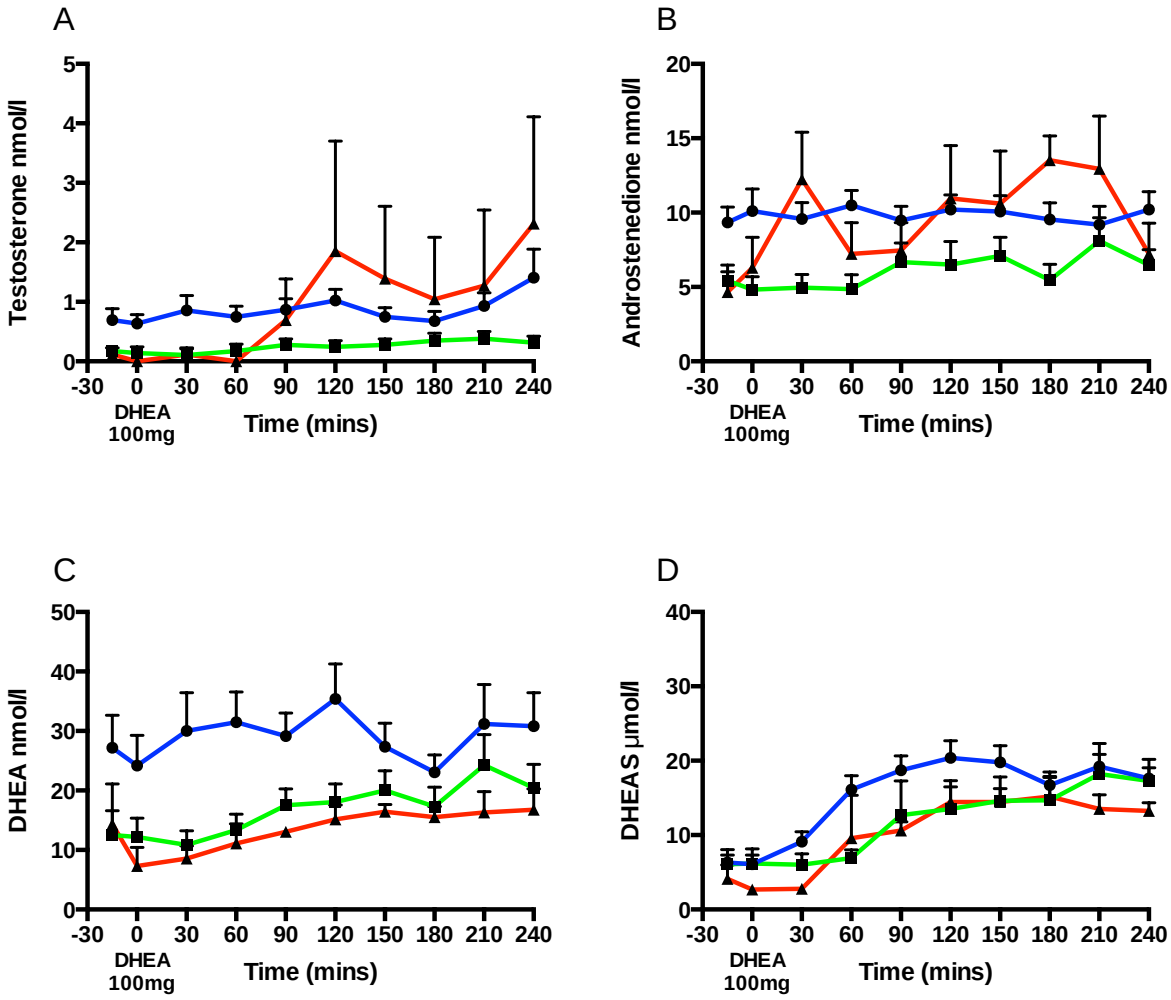


Figure 6-4 Serum testosterone (A), androstenedione (B), DHEA (C) and DHEAS (D) across the DHEA challenge in Protocol B (mixed meal) in healthy controls (green line), PCOS (blue line) and INSR mutation women (red line).

6.3.1.3 Urinary androgens androgen excretion after DHEA exposure

Urinary androgen excretion was measured before and after DHEA in PCOS, control and INSR women in Protocol B (Table 6-2). There were no significant differences in baseline urinary excretion of An, Et, total androgen metabolites (An+Et) or total glucocorticoid metabolites between the three groups, although PCOS had a trend towards higher values than controls. Urinary An, Et and total androgen metabolites (Figure 6-5) increased significantly in all three groups after DHEA. Excretion of An after oral DHEA was higher in INSR compared to controls ($p=0.05$), and there was a trend towards increased excretion compared to PCOS women ($p=0.06$). INSR women had higher urinary levels of Et post-DHEA compared to both PCOS and controls ($p=0.001$ and $p=0.002$, respectively). Urinary excretion of total androgen metabolites post-DHEA was also significantly higher in INSR women compared to both PCOS and controls ($p=0.007$ and $p=0.009$, respectively). Urinary markers of 5α-reductase activity (An/Et and 5α-THF/THF ratios) or total glucocorticoid metabolite excretion did not change significantly from baseline after DHEA in the three study groups (Table 6-2).

Table 6-2 Urinary androgen and glucocorticoid excretion after oral DHEA in controls, PCOS and INSR (Protocol A). Data expressed as mean±SD unless otherwise stated.

a, p<0.05, b, p<0.01, c, p<0.001 as compared to BMI-matched healthy controls. d, p<0.05; e, p<0.01; f, <0.001 as compared to PCOS.

Variable	**Control (n=8)**	**PCOS (n=8)**	**INSR mutations (n=2)**
An pre-DHEA (µg/24h)	1468±977	2369±2375	1537±786
An post-DHEA (µg/24h)	4556±3111	4896±3379	12165±6477[a]
Et pre-DHEA (µg/24h)	1604±1528	1724±1402	1708±1505
Et post-DHEA (µg/24h)	5727±3372	4574±2464	15821±1680[b, e]
Total androgen metabolites (An+Et) pre-DHEA (µg/24h)	3072±2447	4093±3623	3246±2162
Total androgen metabolites post-DHEA (µg/24h)	10283±6254	9471±5722	27986±8157[b, e]
An/Et ratio pre-DHEA (5α-reductase activity)	1.1±0.3	1.4±0.6	1.2±0.6
An/Et ratio post-DHEA (5α-reductase activity)	0.7±0.4	1.0±0.4	0.8±0.3
5α-THF/THF pre-DHEA (5α-reductase activity)	0.8±0.3	1.1±0.6	1.0±0.7
5α-THF/THF post-DHEA (5α-reductase activity)	0.8±0.2	0.9±0.8	0.9±0.8
Total glucocorticoid metabolites pre-DHEA (µg/24h)	4864±2757	6098±3535	3789±667
Total glucocorticoid metabolites post-DHEA (µg/24h)	3694±2225	5581±4742	6042±3264

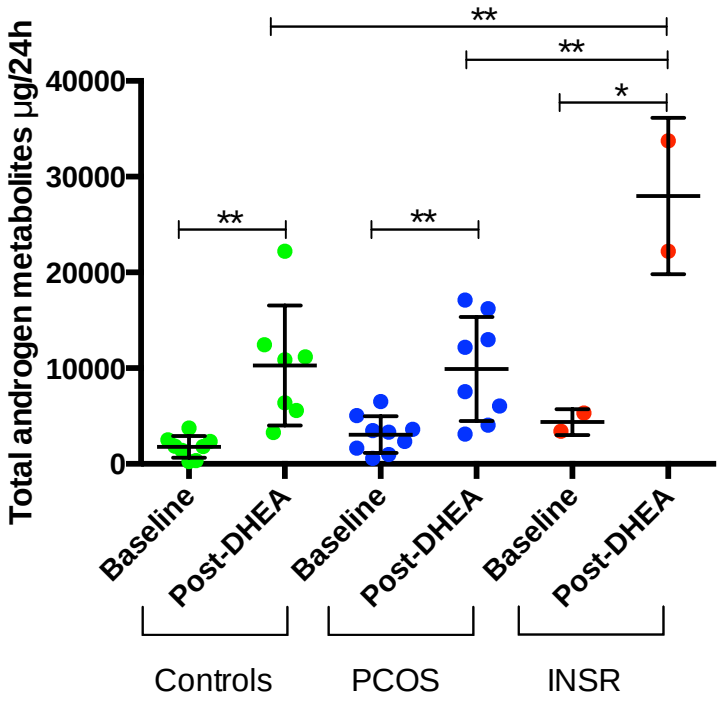


*Figure 6-5 Total urinary androgen metabolite (androsterone + etiocholanolone) excretion after oral DHEA in controls, PCOS and INSR women. Excretion increased significantly in all three groups. INSR women had higher post-DHEA levels compared to both controls and PCOS women. *p<0.05; **p<0.01.*

6.3.1.4 Adipose tissue interstitial fluid androgens pre- and post-DHEA exposure

T and DHT levels in adipose interstitial fluid before and after DHEA in the three study groups are shown in Figure 6-6. Adipose T levels (mean±SEM) were higher in PCOS women compared to controls, both at baseline (4.5±1.9 v 0.1±0.1nmol/l, p=0.03) and after DHEA (5.4±2.5 v 0.1±0.2, p=0.04). T was undetectable in adipose fluid pre- and post-DHEA in the INSR group. There was no significant increase in adipose fluid T after oral DHEA in either group (p=0.77 and p=0.63, respectively). DHT was detectable in adipose fluid in all three groups. PCOS women had a trend towards higher baseline DHT levels than controls (4.5±1.7

v 0.9±0.3nmol/l, p=0.06); post-DHEA levels in the PCOS group were significantly higher than those in controls (5.7±2.1 v 0.6±0.2nmol/l, p=0.03), but comparison of DHT levels pre- and post-DHEA in the PCOS group failed to reach statistical significance (p=0.68). DHT was detectable in INSR adipose fluid both pre- (1.8±0.1nmol/l) and post-DHEA (1.6±0.2nmol/l), with no significant difference between pre- and post-values (p=0.42). Baseline adipose T levels correlated with AUC for serum T (R=0.58, p<0.001) and A (R=0.44, p=0.005) across the DHEA challenge. Baseline adipose DHT levels correlated significantly with the urinary An/Et ratio (R=0.51, p=0.02, Figure 6-7). No association was observed between baseline DHT and the 5α-THF/THF ratio (R=0.33, p=0.15).

Adipose DHEA levels were higher in PCOS women compared to controls pre- (4.4±1.8 v 0.2±0.1nmol/l, p=0.03) and post-DHEA (5.1±2.3 v 0.1±0.1, p=0.04), with no significant differences observed between pre- and post-DHEA values in either group. Androstenedione was also higher in PCOS compared to controls, both before (3.7±1.5 v 0.5±0.1nmol/l, p=0.04) and after (4.2±1.9 v 0.3±0.1, p=0.05) DHEA intake.

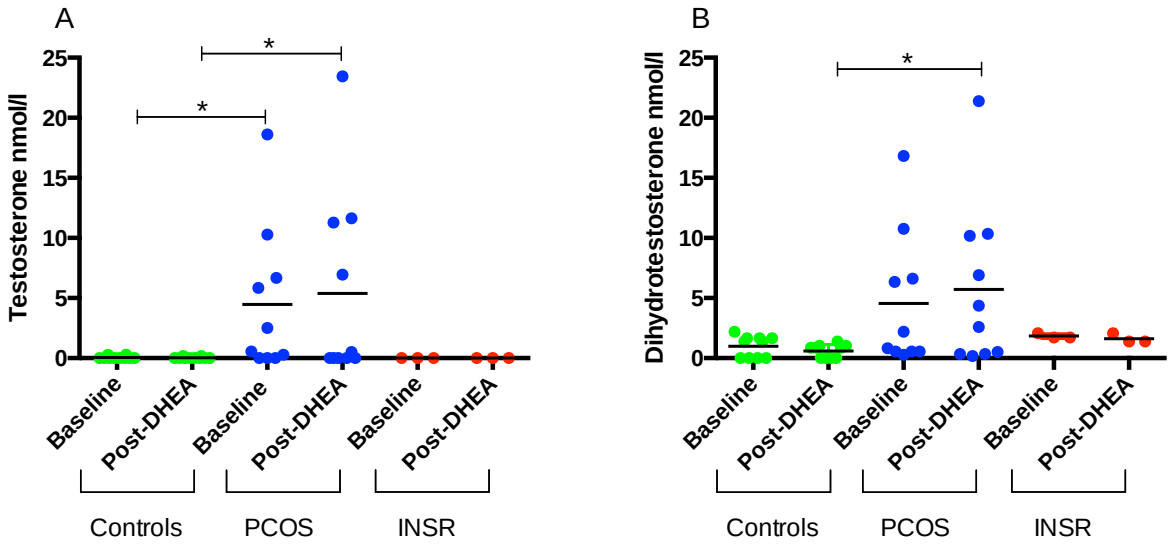


Figure 6-6 T (A) and DHT (B) measured by LC-MS/MS in adipose interestitial fluid pre- and post-DHEA in the three study groups.

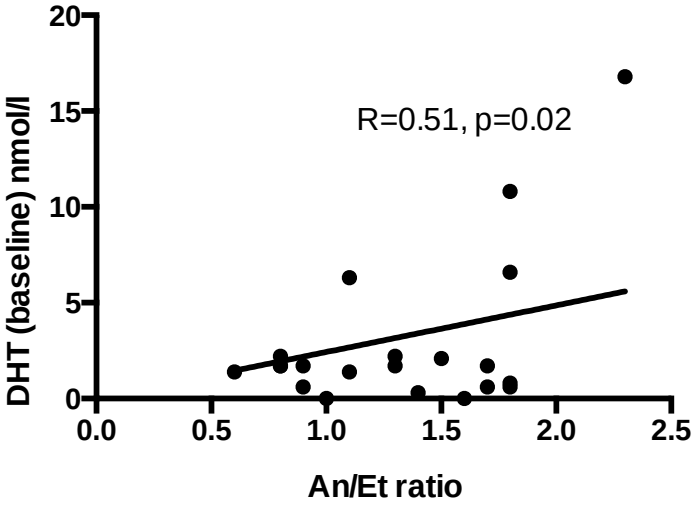


Figure 6-7 Positive relationship between adipose interstitial DHT levels and urinary An/Et ratio (marker of 5α-reductase activity) across all three groups.

6.3.2 Metabolic consequences of DHEA exposure

6.3.2.1 Adipose tissue metabolism in vivo

Realtime markers of adipose insulin sensitivity and lipid metabolism in the three groups (fasted studies only) during adipose microdialysis are highlighted in Figure 6-8. AUC values for pyruvate, lactate and glucose did not differ significantly between control, PCOS and INSR women. AUC for glycerol (mean±SEM μM*min) was significantly higher in INSR than in both PCOS and control patients (123922±31357 [INSR] v 50380±8910 [PCOS], p=0.01; v 70192±9403 [control], p=0.05). AUC for glycerol did not differ significantly between PCOS and controls. However, when analysed separately, AUC values for glycerol (mean±SEM μM*min) were significantly higher in controls compared to PCOS women at 120-249 minutes (35347±4781 v 22310±3577, p=0.04).

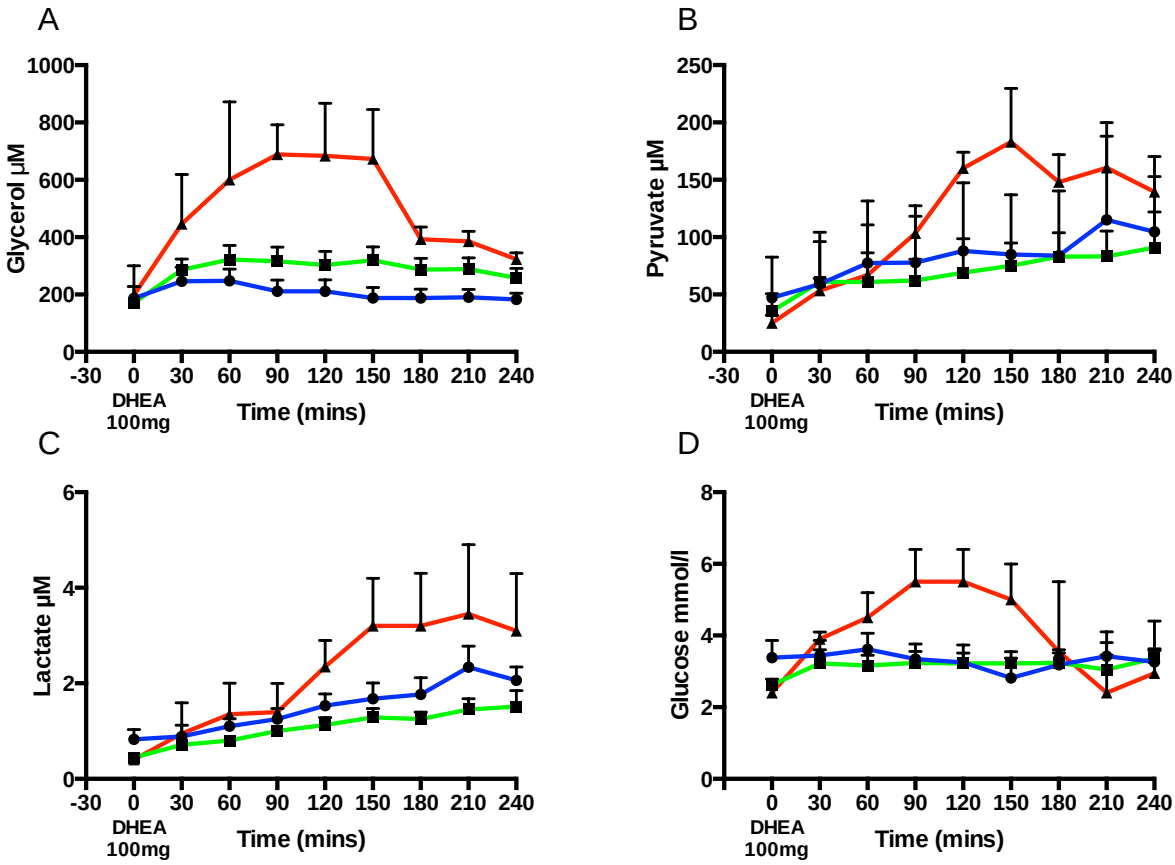


Figure 6-8 Adipose fluid glycerol (A), pyruvate (B), lactate (C) and glucose (D) across the DHEA in healthy controls (n=8, green line), PCOS (n=8, blue line) and INSR mutation women (n=2, red line). Patients remained fasted for the duration of the challenge (Protocol A). Adipose glycerol levels in INSR women are signifcantly elevated compared to the other two groups, consistent with severe insulin resistance. Adipose glycerol AUC between 120 and 240 minutes is significantly lower in PCOS women compared to controls (p=0.04), consistent with relative suppression of lipolysis in the PCOS group.

6.3.2.2 Serum metabolic markers after DHEA exposure

Serum insulin, glucose and FFAs across the DHEA challenge (fasted studies only) are shown in Figure 6-9. Fasting insulin levels were significantly higher in INSR women compared to both controls and PCOS women (1089.7±686.0 [INSR] v 45.1±45.9pmol/l [controls], p=0.005; v 79.8±23.4pmol/l [PCOS], p=0.006. There was no significant difference between fasting insulin levels between PCOS and control women (p=0.30). Serum insulin values did not change significantly over the course of the DHEA challenge in the three study groups. AUC for insulin (mean±SEM pmol/l*min) across the DHEA challenge was significantly higher in INSR women compared to both controls and PCOS women (200759±156888 [INSR] v 8043±2958 [controls], p=0.01; v 14672±4120 [PCOS], p=0.02. AUC for insulin did not differ significantly between PCOS and controls although there was a trend towards increased AUC in PCOS women (14672±4120 v 8043±2958, p=0.21).

Plasma glucose values remained consistent across the DHEA challenge in all three groups (fasted state only). AUC values for glucose (mean±SEM mmol/l*min) were similar in PCOS and control women (1169±30 [controls] v 1173±23 [PCOS], p=0.93). AUC for glucose in the INSR group (1105±4.2) did not differ significantly from PCOS (p=0.20) or controls (p=0.33). AUC for serum FFA (mean±SEM μM*min) after DHEA did not differ significantly between PCOS and controls (341515±76306 [PCOS] v 337401±94454, p=0.93). Mean AUC for INSR patients was 333079±14917, and did not differ significantly from controls (p=0.95) or controls (p=0.88).

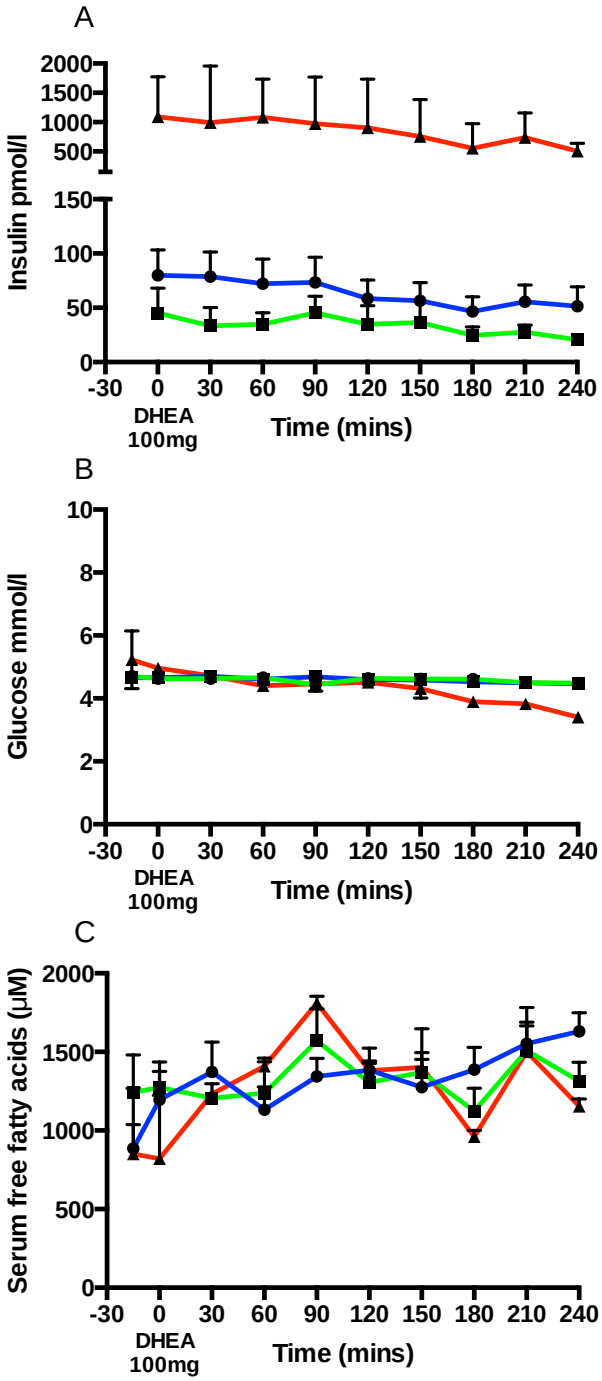


Figure 6-9 Serum insulin (A), glucose (B) and free fatty acids (C) across the DHEA challenge test (fasted patients only, Protocol A) in healthy controls (n=8, green line), PCOS (n=8, blue line) and INSR mutations (n=2, red line). Fasting and AUC values for insulin are highest in INSR patients.

6.3.3 Body composition and androgen metabolism

Body composition results as measured by DXA are shown in Table 6-3. BMI did not differ significantly between the three study groups. No significant differences in android or gynecoid fat percentage, or in the android:gynaecoid ratio, were observed across the three groups, although PCOS women had a trend towards an increased android fat percentage and increased android:gynaecoid ratio compared to controls (p=0.12 and p=0.09, respectively). However, PCOS women had higher visceral fat volume (p=0.002), visceral fat mass (p=0.002) and percentage visceral fat mass (p=0.003) compared to healthy controls. No significant differences in visceral adiposity were detected in INSR women compared to the other two groups. Percentage visceral adiposity correlated positively with HOMA-IR (p=0.002), serum androstenedione (p=0.04), An/Et ratio (p=0.02) and the 5α-THF/THF ratio p=0.02), Table 6-4.

Table 6-3 Markers of visceral adiposity on DXA imaging in all study patients (Protocol A and B). Data expressed as mean±SD unless otherwise stated. a, p<0.05, b, p<0.01, c, p<0.001 as compared to BMI-matched healthy controls.

	Controls (n=18)	**PCOS (n=18)**	**INSR (n=3)**
BMI (kg/m^2)	27.1±5.1	31.9±7.5	29.1±2.5
Android fat (%)	39.5±11.3	47.5±13.2	54.6±3.5
Gynaecoid fat (%)	43.9±5.6	45.6±9.4	53.1±7.6
Android:Gynaecoid fat ratio	0.88±0.20	1.02±0.19	1.10±0.13
Visceral fat volume (cm^3)	413±396	1034±626[b]	841±268
Visceral fat mass (g)	389±374	976±591[b]	793±253
% Visceral fat (of total fat mass)	1.2±0.9	2.4±1.1[b]	2.2±1.0

*Table 6-4 Association between visceral adiposity, insulin sensitivity and androgen metabolism (R-values) across all patients (n=39) using Spearman's correlation testing. *p<0.05; **p<0.01; ***p<0.001.*

	Visceral fat volume	**Visceral fat mass**	**% visceral fat**	**HOMA-IR**	**Serum T**	**Serum A**	**An/Et ratio**	**5α-THF/THF ratio**	**Total androgen metabolites**
Visceral fat volume	-	0.99***	0.94***	0.45**	0.26	0.30	0.48**	0.45**	0.28
Visceral fat mass	0.99***	-	0.94***	0.45**	0.26	0.31	0.48**	0.45**	0.29
% visceral fat	0.94***	0.94***	-	0.49**	0.26	0.33*	0.38*	0.37*	0.28
HOMA-IR	0.45**	0.45**	0.49**	-	0.28	0.41*	0.35*	0.33*	0.31
Serum T	0.26	0.26	0.26	0.28	-	0.43**	0.34*	0.29	0.33*
Serum A	0.30	0.31	0.33*	0.41*	0.43**	-	0.48**	0.32*	0.21
An/Et ratio	0.48**	0.48**	0.38*	0.35*	0.34*	0.48**	-	0.76***	0.05
5α-THF/THF ratio	0.45**	0.45**	0.37*	0.33*	0.29	0.32*	0.76***	-	0.12
Total androgen metabolites	0.28	0.29	0.28	0.31	0.33*	0.21	0.05	0.12	-

6.4 Discussion

The *in vivo* findings presented in this chapter highlight the role of adipose tissue as an important organ of peripheral androgen activation in PCOS, and support many of the *in vitro* data from chapter 4. Intra-adipose androgens were detectable in microdialysis fluid in a subset of women with PCOS, whilst present only in trivial amounts in the other two groups. DHT, the most potent androgen, was neither quantifiable nor detectable in the serum of study participants, even after DHEA, on our current LC-MS/MS assay, and therefore its detection in microdialysis fluid in SC fat robustly endorses a role for adipose tissue in androgen metabolism in PCOS. Adipose microdialysis has also facilitated the study of real-time effects of acute androgen exposure on lipid metabolism *in vivo*, with relative suppression of lipolysis demonstrated in PCOS women after DHEA compared to healthy controls. These data indirectly corroborate our *de novo* lipogenesis results from chapter 5, in which T and DHT promoted lipid accumulation in SC adipocytes. The utility of the DHEA challenge test in the investigation of *in vivo* androgen metabolism in humans is highlighted by the dramatic increases in serum and urinary androgen metabolites in study participants after DHEA administration, consistent with previous studies (Fassnacht et al., 2003). The observed changes in intra-adipose glycerol levels after DHEA also underpin its role as a tool for the study of acute androgen-mediated effects in human biology.

Evidence for intra-adipose androgen generation is convincing from multiple previous studies (Quinkler et al., 2004, Blouin et al., 2003). Local generation of androgens by adipocytes is particularly intriguing in the context of PCOS-related metabolic dysfunction. Adipose tissue plays an integral role in human energy homeostasis and metabolism (Tomlinson et al., 2008a), and recent studies, including our own data from chapter 5, have highlighted that androgens, acting via the AR, induce changes in adipocyte size and energy-

storing capacity (Caldwell et al., 2015). We have previously discussed how chronic DHT exposure in female mice, whether *in utero* or at later developmental stages, induces adipocyte hypertrophy and metabolic derangements (Caldwell et al., 2014, Yan et al., 2013). Increased adipocyte size has been intimately linked to insulin resistance, dyslipidaemia and hypertension (Vazquez-Vela et al., 2008), and appears to be more metabolically deleterious than the fat expansion observed in hyperplasia (Villa and Pratley, 2011). Cumulatively, these observations suggest that adipose-specific androgen generation may fuel an adverse metabolic phenotype in PCOS, creating a vicious circle which may be driven by hyperinsulinaemia. Unfortunately, the numbers in our study were too small to meaningfully correlate adipose DHT levels with markers of systemic insulin resistance or abdominal obesity in the PCOS group. To our knowledge, this is the first attempt to measure intra-adipose androgen concentration by LC-MS/MS in human microdialysis fluid. T generation was previously measured by thin layer chromatography (TLC) in differentiated SC and OM adipocytes in the study by Quinkler *et al*, who showed increased conversion of A to T in the SC samples. Glucocorticoids have been assayed using LC-MS/MS in adipose microdialysis fluid from both rats and monkeys (Huang et al., 2011, Sun et al., 2008), while E2 levels have been measured in SC and OM samples from severely obese patients (Wang et al., 2013). Detection of adipose androgens by LC-MS/MS now raises the exciting prospect of tissue-specific sex hormone estimation in future studies, including in androgen deficiency syndromes in men.

We demonstrated relative suppression of adipose lipolysis, as measured by microdialysis fluid glycerol concentrations, in PCOS women after DHEA, compared to healthy controls. This would appear to support our *in vitro* observations of upregulated ACC1 expression and enhanced *de novo* lipogenesis in androgen-exposed SGBS cells (sections 5.3.2.1 and 5.3.2.2). Recent studies have also demonstrated enhanced lipogenesis in response

to DHT in primary human female hepatocytes, a finding not observed in male tissue (Nasiri et al., 2015); Varlamov and colleagues also found attenuation of lipolysis, increased FAS mRNA expression and elevated FFA uptake in visceral WAT from females exposed to chronic androgen excess (Varlamov et al., 2013). Interestingly, one study suggested significant metabolic heterogeneity between obese and non-obese women with PCOS using a nontargeted GC/MS serum metabolomics approach, demonstrating increased lipolysis, likely secondary to insulin resistance, in obese women, and, conversely, suppression of lipolysis, likely secondary to significant androgen excess, in the non-obese cohort (Escobar-Morreale et al., 2012). Once again the small numbers in this study did not allow us to analyse obese and non-obese patients separately, as only 3 patients out of 8 had a BMI less than 30kg/m^2 in the PCOS group.

A weight-loss intervention in PCOS women, who had similar age and weight parameters to those in the study above (mean BMI 32.0±1.6kg/m^2), revealed reduced baseline catecholamine-stimulated lipolysis in SC fat in PCOS compared to controls using adipose microdialysis (Moro et al., 2009). This resistance of lipolysis to catecholamine stimulation subsequently improved after weight loss, despite the absence of changes in adipose insulin sensitivity. This suggests that the defects in lipolysis occur independent of insulin resistance. This is supported by our finding of constant serum insulin, glucose and FFA levels across the DHEA challenge in all groups, with no significant change in response to *in vivo* androgen exposure (in contrast to the *in vitro* observations in chapter 5). Androgen-mediated lipogenesis and adipocyte hypertrophy could represent a driving force for fat mass expansion and weight gain in PCOS. Reduction of androgen burden with pharmacotherapy, coupled with lifestyle intervention, may therefore represent the best strategy to enhance lipolysis and induce weight loss in PCOS.

Comparison of serum androgens from the DHEA challenge test in the fed and fasted state does not suggest a major role for caloric intake in androgen metabolism in women. Interpretation of these results, however, is limited by the absence of a crossover component to the study (with the exception of the INSR women), whereby the same patients would have completed both protocols to allow direct comparison. AUC and baseline T values in the DHEA challenge were similar between fed and fasted PCOS women, and between fed and fasted controls. AUC for A was significantly higher in the fed PCOS and control groups compared to their fasted counterparts; however, higher baseline A levels in the fed study suggests that these were androgenically distinct from the fasted groups even before caloric intake. This baseline difference in the two PCOS groups underpins many of the difficulties with the interpretation of studies in this disorder, by highlighting the presence of significant biochemical and metabolic heterogeneity, despite methodical BMI matching of all study groups. Serum A and T in the hyperinsulinaemic INSR cohort after DHEA appear to be significantly higher than in the other two groups, suggesting a major role for insulin in androgen generation, although statistical analysis is not likely to be informative in view of the small numbers.

Our observation of increased markers of visceral adiposity in the PCOS group compared to BMI-matched controls is interesting, but these findings have not been replicated in all studies of fat distribution in PCOS. Discrepancies in results of studies on fat distribution in PCOS are often attributable to the use of distinct imaging modalities, or to heterogeneity in study groups as described above. Barber and colleagues found that PCOS and BMI-matched controls were indistinguishable in terms of visceral, abdominal, SC and gluteofemoral fat distribution on axial MRI imaging (Barber et al., 2008). A recent study employing ultrasound found that PCOS women had increased intra-abdominal and mesenteric fat thickness

compared to BMI-matched controls, representing an 'intermediate' phenotype between men and women (Borruel et al., 2013). PCOS women also had increased global adiposity compared to controls. Using a composite of anthropometric markers (waist circumference) and lipid biochemistry to calculate validated indices of adiposity, Tehrani and colleagues found no difference in the visceral adiposity index (VAI) between PCOS and controls, but noted significantly higher lipid accumulation product (LAP) values in PCOS women (Ramezani Tehrani et al., 2014). In the DXA data presented in this chapter, we found no significant differences in android or gynecoid fat distribution between PCOS and controls, but noted increased visceral fat mass and volume, and increased visceral fat percentage of total fat mass, in the PCOS group. Visceral adiposity was positively associated with serum androgens, systemic 5α-reductase activity, and markers of insulin resistance, suggesting that increased visceral fat in PCOS is a surrogate marker of circulating androgen burden and metabolic disease.

The clinical, biochemical and radiological data presented in this chapter support our overriding hypothesis of intra-adipose androgen generation, abdominal obesity and insulin resistance in PCOS. Using state-of-the-art *in vivo* research tools and biochemical analytical techniques, as well as diagnostic challenge test, we have identified key differences in serum, urinary and adipose androgen metabolism in PCOS, control and INSR mutation women, and have correlated these derangements with systemic insulin resistance and markers of adiposity. The summary and final conclusions of this thesis will consider all the data presented and set forth a clear strategy for future research directions in this field.

7 Final conclusions and future directions

7.1 Final conclusions

PCOS is a complex, heterogeneous disorder, manifesting clinically with a series of diverse phenotypes. As our understanding of PCOS evolves, it is increasingly apparent that it represents the common clinical endpoint of a number of biologically distinct metabolic disturbances, and that current diagnostic and classification strategies, as discussed in section 1.2.1, are largely inadequate. The notion that PCOS is a disorder of primary ovarian origin appears increasingly outdated. However, obesity, androgen excess and insulin resistance are highly prevalent in PCOS, are present in the majority of patients, and their respective phenotypic severities are closely correlated (O'Reilly et al., 2014a). This suggests that all three abnormalities are intimately linked, and play a causative role in the pathogenesis of PCOS, at least in the majority of patients. On the basis of the data presented in this thesis, we believe that adipose tissue is a crucial site of interaction between insulin signaling and androgen metabolism in women, functioning as an intracrine organ of androgen activation, as well as a target of androgen action. We have presented evidence for pro-lipogenic effects of androgens at cellular level, resulting in adipocyte hypertrophy, fat accumulation and insulin resistance. Secondary hyperinsulinaemia may fuel this vicious circle by further stimulating local adipose androgen generation through upregulation of AKR1C3 expression and activity (Figure 7-1). Further work is needed to understand if these mechanisms are only relevant in the context of PCOS-related obesity. Adipose androgens were detectable in only a subset of PCOS in the clinical study, although interestingly levels did not correlate with BMI or radiological indices of adiposity.

The rationale for our interest in adipose tissue as a key player in androgen metabolism is based upon the association of hyperandrogenism with adiposity in females (Mongraw-Chaffin et al., 2015, Borruel et al., 2013). Intriguingly, this correlation appears to contrast

with that observed in men, where abdominal obesity and metabolic dysfunction are associated with low levels of circulating sex hormones. The role of peripheral tissue in androgen activation has been recognized for many years (Stewart et al., 1990, Fassnacht et al., 2003), and the persistence of hyperandrogenism after pharmacological suppression of ovarian and adrenal androgen output suggests that this phenomenon is very much physiologically relevant (Rosenfield et al., 2011). Adipose tissue expresses a number of androgen-metabolising enzymes, and expression in women correlates with clinical markers of obesity such as BMI and waist circumference (Quinkler et al., 2004, Wake et al., 2007). As adipocytes possess the necessary cellular machinery for androgen activation and secretion (Blanchette et al., 2005, Blouin et al., 2009a), it seems very reasonable to hypothesise that obesity increases local and systemic androgen burden. AKR1C3, which converts the weak androgen precursor A into potent T, is highly expressed in adipose tissue, particularly in the SC fat depot. AKR1C3 expression is higher in SC tissue from PCOS women compared to weight-matched controls (Wang et al., 2012). We found a strong correlation between expression and BMI in 38 women. Interestingly, expression of the androgen-inactivating isoform, AKR1C2, also increased with obesity, suggesting the possibility of physiological equilibrium in adipose androgen metabolism in simple obesity. Insulin increased AKR1C3 expression and activity in both primary female SC adipocytes and SGBS cells *in vitro*, and although its effect on AKR1C2 was not explored, it is possible that hyperinsulinaemia, in the setting of systemic insulin resistance, is sufficient to tip the scales in favour of adipose androgen activation in PCOS.

In the clinical setting, the association of circulating androgens with insulin resistance has been explored in multiple studies, but none to our knowledge have attempted to delineate metabolic risk by simultaneous measurement of T and A using LC-MS/MS. In the study

presented in chapter 3, the utility of serum A as a marker of insulin resistance is demonstrated by its inverse relationship with the OGTT-derived ISI; furthermore, those PCOS women with co-elevation of both A and T above the normal reference range had the lowest ISI and highest HOMA-IR values, as well as an increased prevalence of dysglycaemia (O'Reilly et al., 2014b). We believe that this strategy should be adapted into routine clinical practice in PCOS as it is a relatively straightforward screening tool to identify those at high risk of overt dysglycaemia and, by inference, future cardiovascular disease. These data notwithstanding, obesity remains the biggest risk factor for progression to frank hyperglycaemia in PCOS (Azziz et al., 2006), and obese PCOS women have an increased risk of progression to overt diabetes compared to control women with simple obesity (Jayasena and Franks, 2014). This suggests that obesity-related factors specific to PCOS may be responsible for β-cell decompensation and onset of hyperglycaemia. Adipose-specific androgen excess in obese PCOS women, fuelled by hyperinsulinaemia, may aggravate metabolic disturbances by impacting directly on local adipocyte function, and lead to acceleration of β-cell failure due to the insulinotropic effects of androgens, as highlighted in chapter 5.

Our *in vivo* and *in vitro* results support an emerging body of animal and human data on the role of androgens in adipose lipid accumulation. In SGBS cells, T and DHT upregulated ACC1 mRNA expression, and this correlated with increased rates of *de novo* lipogenesis as measured by uptake of 1-[^{14}C]-acetate into the cellular component as lipid. Androgens also increased insulin-stimulated glucose uptake and suppressed β-oxidation, with a net effect of increased intracellular lipid accumulation. The use of primary fat culture for *in vitro* adipocyte studies, taken from distinct fat depots from both male and female patients, where available, is always preferable to the use of immortalized cell lines. However, SGBS cells have been characterised as a model for the study of human adipocyte biology (Wabitsch

et al., 2001), and we were able to validate their utility in the study of lipid metabolism by showing typical morphological changes and upregulation of lipogenic genes across differentiation. In our clinical study, PCOS women had relative suppression of lipolysis, measured indirectly by intra-adipose glycerol concentrations, compared to healthy controls after administration of the androgen precursor DHEA, and glycerol levels were inversely proportional to serum androgen generation at equivalent time points. This is compelling *in vivo* corroboration of our *in vitro* work showing enhanced adipose lipogenesis after androgen exposure. Androgens, acting via the AR, appear to induce lipogenesis by binding to the androgen response elements in the SREBP family of transcription factors (Zhou et al., 2012). SREBP-1 regulated genes in the fatty acid biosynthesis pathway, while SREBP-2 plays a role in cholesterol metabolism (Shimano et al., 1999). It is fascinating that androgen-mediated upregulation of SREBP-1a and SREBP-1c mRNA and protein expression drives excess lipid production and secretion in epidermal keratinocytes and sebaceous glands, contributing significantly to dermatological diseases such as acne vulgaris and seborrhoeic dermatitis (Rosignoli et al., 2003). The lipogenic actions of androgens also play a crucial role in growth and survival of prostate cancer cells (Huang et al., 2012). However, the contribution of pro-lipogenic effects of androgens in adipose tissue to metabolic disease is an area which has until now been largely neglected.

Detection of significant concentrations of T and DHT in adipose microdialysis fluid emphasises the important of fat as an organ of androgen activation. It is also exciting to consider that measured T concentrations are likely to reflect free rather than ‘bound’ steroid levels. SHBG circulates as a homodimer, consisting of two monomers of 43.7kDa each (Grishkovskaya et al., 2000); the microdialysis membrane filtration cut-off for molecules is set at 20kDa or below. It is therefore highly likely that any T detectable in adipose fluid is

representative of unbound, physiologically active T. DHT was undetectable in serum, yet was quantifiable in adipose fluid, and correlated with systemic 5α-reductase activity. More sensitive LC-MS/MS assays for serum DHT measurement will be a crucial factor in our future studies on PCOS steroidomics, and its quantification in serum has already proved useful in predicting metabolic dysfunction (Munzker et al., 2015). In a subset of our PCOS patients, adipose DHT levels were as high as 20nmol/l, highlighting the significant steroidogenic and metabolic heterogeneity in this disorder. Small patient numbers rendered any further statistical correlation relatively meaningless, but it will be interesting in the future to examine potential associations with BMI, fat distribution and insulin resistance.

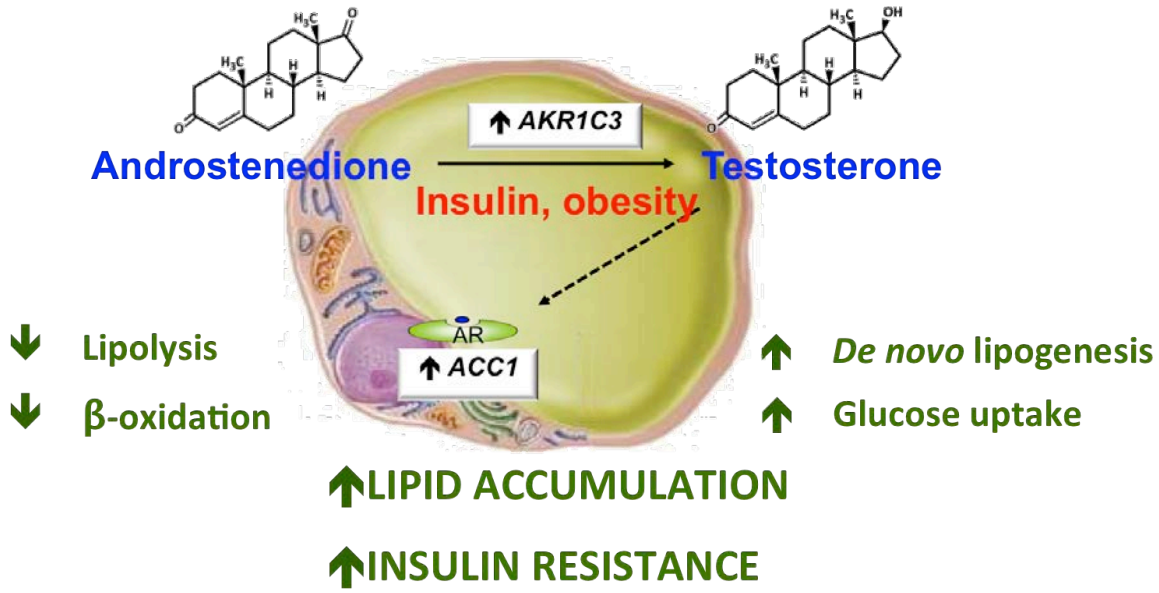


Figure 7-1 The vicious circle of adipose androgen activation, adipocyte hypertrophy and insulin resistance in PCOS.

In summary, we have described novel putative mechanisms by which androgens may regulate adipose tissue function, fat mass and lipid metabolism. A vicious of circle of adipose androgen activation, lipid accumulation and increased fat mass may drive systemic insulin resistance, with androgen generation in fat further exacerbated by hyperinsulinaemia (Figure 7-1). Selective inhibition of androgen activation in fat may offer a novel therapeutic strategy to break this cycle and ameliorate the adverse effects of androgen exposure on metabolic phenotype in PCOS.

7.2 Future directions

7.2.1 Depot-specific and gender-specific studies in primary human tissue culture

The work presented in this thesis has highlighted a role for insulin in the promotion of androgen generation in adipose tissue. This appears to be predominantly mediated through its stimulatory effects on AKR1C3 expression in human fat. Although our primary culture data suggest that this process occurs preferentially in SC adipose tissue, sample numbers were small, particularly when SC and OM cultures must be analysed separately. Our data on the effects of androgens on adipose lipid metabolism, whilst robust, are limited by the use of an immortalized cell line. Furthermore, any study on the influence of sex hormones on adipose tissue biology should consider the high likelihood of sexual dimorphism in any observed effects. In the future, therefore, in collaboration with the Tissue Biorepository at the University of Birmingham, we intend to examine the associations observed in this chapter in primary human adipose culture from SC and OM depots in both male and female (pre- and post-menopausal) patients.

7.2.2 Selective inhibition of AKR1C3 activity *in vivo*

AKR1C3 has emerged as an exciting novel therapeutic target for androgen excess in PCOS. The association of adipose androgen activation with lipid accumulation and metabolic disturbance demonstrated in this thesis raises the possibility that selective modulation of AKR1C3 activity could improve both androgen burden and metabolic phenotype. To this end, we have designed a dedicated proof-of-concept clinical study, in which detailed metabolic phenotyping will be carried out in hyperandrogenic PCOS women before and after treatment with the NSAID indomethacin, which is known to have good selective activity against

AKR1C3. Patients will undergo deep phenotyping with hyperinsulinaemic euglycaemic clamp for whole-body insulin sensitivity, as well as selective measurement of adipose vein free fatty acid and androgen levels after ultrasound-guided cannulation.

7.2.3 Metabolomics and the transcriptome

The results presented in this PhD thesis have underscored the close association of hyperandrogenaemia with metabolic disease in women. However, most of the proposed mechanisms need to be examined at the level of the metabolome. With this in mind, we have recently submitted PCOS and control serum, urine and microdialysis fluid samples pre- and post-DHEA exposure for non-targeted metabolomics assessment by high-performance liquid-chromatography/tandem mass spectrometry in the Birmingham MRC Phenome Centre. This will increase our understanding of enriched pathways and metabolite classes in response to an acute androgen load. To verify these data at the level of the adipose transcriptome, we will perform state of the art RNA-sequencing in the School of Biosciences on SC abdominal fat biopsies from PCOS and control women before and after DHEA exposure.

We believe that these proposed in-depth *in vivo* and *ex vivo* studies will increase our understanding of the role of androgens in PCOS-related metabolic dysfunction in the future. Deep phenotyping of PCOS women using *in vivo* metabolic testing, as well as identification of a unique metabolome and transcriptome in these patients, will help to unravel new mechanisms of disease and identify future therapeutic targets for amelioration of androgen burden and metabolic disturbance.

8 References

ABBOTT, D. H., BARNETT, D. K., BRUNS, C. M. & DUMESIC, D. A. 2005. Androgen excess fetal programming of female reproduction: a developmental aetiology for polycystic ovary syndrome? *Hum Reprod Update,* 11**,** 357-74.

ABUMRAD, N., COBURN, C. & IBRAHIMI, A. 1999. Membrane proteins implicated in long-chain fatty acid uptake by mammalian cells: CD36, FATP and FABPm. *Biochim Biophys Acta,* 1441**,** 4-13.

ABUMRAD, N. A. & DAVIDSON, N. O. 2012. Role of the gut in lipid homeostasis. *Physiol Rev,* 92**,** 1061-85.

ADASHI, E. Y., HSUEH, A. J. & YEN, S. S. 1981. Insulin enhancement of luteinizing hormone and follicle-stimulating hormone release by cultured pituitary cells. *Endocrinology,* 108**,** 1441-9.

AILHAUD, G., DANI, C., AMRI, E. Z., DJIAN, P., VANNIER, C., DOGLIO, A., FOREST, C., GAILLARD, D., NEGREL, R. & GRIMALDI, P. 1989. Coupling growth arrest and adipocyte differentiation. *Environ Health Perspect,* 80**,** 17-23.

ALEXANDERSON, C., ERIKSSON, E., STENER-VICTORIN, E., LYSTIG, T., GABRIELSSON, B., LONN, M. & HOLMANG, A. 2007. Postnatal testosterone exposure results in insulin resistance, enlarged mesenteric adipocytes, and an atherogenic lipid profile in adult female rats: comparisons with estradiol and dihydrotestosterone. *Endocrinology,* 148**,** 5369-76.

AMRI, E. Z., VANNIER, C., ETIENNE, J. & AILHAUD, G. 1986. Maturation and secretion of lipoprotein lipase in cultured adipose cells. II. Effects of tunicamycin on activation and secretion of the enzyme. *Biochim Biophys Acta,* 875**,** 334-43.

ANDERSON, D. C. 1974. Sex-hormone-binding globulin. *Clinical endocrinology,* 3**,** 69-96.

ANDERSON, H., FOGEL, N., GREBE, S. K., SINGH, R. J., TAYLOR, R. L. & DUNAIF, A. 2010. Infants of women with polycystic ovary syndrome have lower cord blood androstenedione and estradiol levels. *J Clin Endocrinol Metab,* 95**,** 2180-6.

ANDREW, R., PHILLIPS, D. I. & WALKER, B. R. 1998. Obesity and gender influence cortisol secretion and metabolism in man. *J Clin Endocrinol Metab,* 83**,** 1806-9.

ANDROULAKIS, II, KANDARAKI, E., CHRISTAKOU, C., KARACHALIOS, A., MARINAKIS, E., PATERAKIS, T. & DIAMANTI-KANDARAKIS, E. 2014. Visceral adiposity index (VAI) is related to the severity of anovulation and other clinical features in women with polycystic ovary syndrome. *Clin Endocrinol (Oxf),* 81**,** 426-31.

APTER, D., BUTZOW, T. L., LAUGHLIN, G. A. & YEN, S. S. 1993. Gonadotropin-releasing hormone pulse generator activity during pubertal transition in girls: pulsatile and diurnal patterns of circulating gonadotropins. *J Clin Endocrinol Metab,* 76**,** 940-9.

ARLT, W. 2004. Dehydroepiandrosterone and ageing. *Best Pract Res Clin Endocrinol Metab,* 18**,** 363-80.

ARLT, W., CALLIES, F., VAN VLIJMEN, J. C., KOEHLER, I., REINCKE, M., BIDLINGMAIER, M., HUEBLER, D., OETTEL, M., ERNST, M., SCHULTE, H. M.

& ALLOLIO, B. 1999. Dehydroepiandrosterone replacement in women with adrenal insufficiency. *N Engl J Med,* 341**,** 1013-20.

ARLT, W., WALKER, E. A., DRAPER, N., IVISON, H. E., RIDE, J. P., HAMMER, F., CHALDER, S. M., BORUCKA-MANKIEWICZ, M., HAUFFA, B. P., MALUNOWICZ, E. M., STEWART, P. M. & SHACKLETON, C. H. 2004. Congenital adrenal hyperplasia caused by mutant P450 oxidoreductase and human androgen synthesis: analytical study. *Lancet,* 363**,** 2128-35.

ARLT, W., WILLIS, D. S., WILD, S. H., KRONE, N., DOHERTY, E. J., HAHNER, S., HAN, T. S., CARROLL, P. V., CONWAY, G. S., REES, D. A., STIMSON, R. H., WALKER, B. R., CONNELL, J. M., ROSS, R. J. & UNITED KINGDOM CONGENITAL ADRENAL HYPERPLASIA ADULT STUDY, E. 2010. Health status of adults with congenital adrenal hyperplasia: a cohort study of 203 patients. *J Clin Endocrinol Metab,* 95**,** 5110-21.

ARSLANIAN, S. A., LEWY, V., DANADIAN, K. & SAAD, R. 2002. Metformin therapy in obese adolescents with polycystic ovary syndrome and impaired glucose tolerance: amelioration of exaggerated adrenal response to adrenocorticotropin with reduction of insulinemia/insulin resistance. *J Clin Endocrinol Metab,* 87**,** 1555-9.

ASHCROFT, F. M. & RORSMAN, P. 2012. Diabetes mellitus and the beta cell: the last ten years. *Cell,* 148**,** 1160-71.

ASSCHEMAN, H., GOOREN, L. J., MEGENS, J. A., NAUTA, J., KLOOSTERBOER, H. J. & EIKELBOOM, F. 1994. Serum testosterone level is the major determinant of the male-female differences in serum levels of high-density lipoprotein (HDL) cholesterol and HDL2 cholesterol. *Metabolism,* 43**,** 935-9.

AUCHUS, R. J. & ARLT, W. 2013. Approach to the patient: the adult with congenital adrenal hyperplasia. *J Clin Endocrinol Metab,* 98**,** 2645-55.

AZZIZ, R., CARMINA, E., DEWAILLY, D., DIAMANTI-KANDARAKIS, E., ESCOBAR-MORREALE, H. F., FUTTERWEIT, W., JANSSEN, O. E., LEGRO, R. S., NORMAN, R. J., TAYLOR, A. E., WITCHEL, S. F. & ANDROGEN EXCESS, S. 2006. Positions statement: criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an Androgen Excess Society guideline. *J Clin Endocrinol Metab,* 91**,** 4237-45.

AZZIZ, R., CARMINA, E., DEWAILLY, D., DIAMANTI-KANDARAKIS, E., ESCOBAR-MORREALE, H. F., FUTTERWEIT, W., JANSSEN, O. E., LEGRO, R. S., NORMAN, R. J., TAYLOR, A. E., WITCHEL, S. F., TASK FORCE ON THE PHENOTYPE OF THE POLYCYSTIC OVARY SYNDROME OF THE ANDROGEN, E. & SOCIETY, P. 2009. The Androgen Excess and PCOS Society criteria for the polycystic ovary syndrome: the complete task force report. *Fertil Steril,* 91**,** 456-88.

AZZIZ, R., EHRMANN, D. A., LEGRO, R. S., FERESHETIAN, A. G., O'KEEFE, M., GHAZZI, M. N. & GROUP, P. C. T. S. 2003. Troglitazone decreases adrenal androgen levels in women with polycystic ovary syndrome. *Fertil Steril,* 79**,** 932-7.

AZZIZ, R., MARIN, C., HOQ, L., BADAMGARAV, E. & SONG, P. 2005. Health care-related economic burden of the polycystic ovary syndrome during the reproductive life span. *J Clin Endocrinol Metab,* 90**,** 4650-8.

AZZIZ, R., OCHOA, T. M., BRADLEY, E. L., JR., POTTER, H. D. & BOOTS, L. R. 1995. Leuprolide and estrogen versus oral contraceptive pills for the treatment of hirsutism: a prospective randomized study. *J Clin Endocrinol Metab,* 80**,** 3406-11.

AZZIZ, R., WOODS, K. S., REYNA, R., KEY, T. J., KNOCHENHAUER, E. S. & YILDIZ, B. O. 2004. The prevalence and features of the polycystic ovary syndrome in an unselected population. *The Journal of clinical endocrinology and metabolism,* 89**,** 2745-9.

BAJUK STUDEN, K., JENSTERLE SEVER, M. & PFEIFER, M. 2013. Cardiovascular risk and subclinical cardiovascular disease in polycystic ovary syndrome. *Front Horm Res,* 40**,** 64-82.

BALLARD, F. J. & HANSON, R. W. 1967. Phosphoenolpyruvate carboxykinase and pyruvate carboxylase in developing rat liver. *Biochem J,* 104**,** 866-71.

BARBER, T. M. & FRANKS, S. 2013. Adipocyte biology in polycystic ovary syndrome. *Mol Cell Endocrinol,* 373**,** 68-76.

BARBER, T. M., GOLDING, S. J., ALVEY, C., WASS, J. A., KARPE, F., FRANKS, S. & MCCARTHY, M. I. 2008. Global adiposity rather than abnormal regional fat distribution characterizes women with polycystic ovary syndrome. *The Journal of clinical endocrinology and metabolism,* 93**,** 999-1004.

BARBER, T. M., WASS, J. A., MCCARTHY, M. I. & FRANKS, S. 2007. Metabolic characteristics of women with polycystic ovaries and oligo-amenorrhoea but normal androgen levels: implications for the management of polycystic ovary syndrome. *Clinical endocrinology,* 66**,** 513-7.

BARRY, J. A., AZIZIA, M. M. & HARDIMAN, P. J. 2014. Risk of endometrial, ovarian and breast cancer in women with polycystic ovary syndrome: a systematic review and meta-analysis. *Hum Reprod Update,* 20**,** 748-58.

BARTH, J. H., JENKINS, M. & BELCHETZ, P. E. 1997. Ovarian hyperthecosis, diabetes and hirsuties in post-menopausal women. *Clin Endocrinol (Oxf),* 46**,** 123-8.

BARTH, J. H., YASMIN, E. & BALEN, A. H. 2007. The diagnosis of polycystic ovary syndrome: the criteria are insufficiently robust for clinical research. *Clinical endocrinology,* 67**,** 811-5.

BATTIPROLU, P. K., HOJAYEV, B., JIANG, N., WANG, Z. V., LUO, X., IGLEWSKI, M., SHELTON, J. M., GERARD, R. D., ROTHERMEL, B. A., GILLETTE, T. G., LAVANDERO, S. & HILL, J. A. 2012. Metabolic stress-induced activation of FoxO1 triggers diabetic cardiomyopathy in mice. *J Clin Invest,* 122**,** 1109-18.

BAUMAN, D. R., RUDNICK, S. I., SZEWCZUK, L. M., JIN, Y., GOPISHETTY, S. & PENNING, T. M. 2005. Development of nonsteroidal anti-inflammatory drug analogs and steroid carboxylates selective for human aldo-keto reductase isoforms: potential antineoplastic agents that work independently of cyclooxygenase isozymes. *Mol Pharmacol,* 67**,** 60-8.

BELANGER, C., HOULD, F. S., LEBEL, S., BIRON, S., BROCHU, G. & TCHERNOF, A. 2006. Omental and subcutaneous adipose tissue steroid levels in obese men. *Steroids,* 71**,** 674-82.

BELLEMARE, V., LABERGE, P., NOEL, S., TCHERNOF, A. & LUU-THE, V. 2009. Differential estrogenic 17beta-hydroxysteroid dehydrogenase activity and type 12 17beta-hydroxysteroid dehydrogenase expression levels in preadipocytes and differentiated adipocytes. *J Steroid Biochem Mol Biol,* 114**,** 129-34.

BENNETT, N. C., GARDINER, R. A., HOOPER, J. D., JOHNSON, D. W. & GOBE, G. C. 2010. Molecular cell biology of androgen receptor signalling. *Int J Biochem Cell Biol,* 42**,** 813-27.

BERGH, C., CARLSSON, B., OLSSON, J. H., SELLESKOG, U. & HILLENSJO, T. 1993. Regulation of androgen production in cultured human thecal cells by insulin-like growth factor I and insulin. *Fertil Steril,* 59**,** 323-31.

BERRY, D. C., DESANTIS, D., SOLTANIAN, H., CRONIGER, C. M. & NOY, N. 2012. Retinoic acid upregulates preadipocyte genes to block adipogenesis and suppress diet-induced obesity. *Diabetes,* 61**,** 1112-21.

BIDDINGER, S. B. & KAHN, C. R. 2006. From mice to men: insights into the insulin resistance syndromes. *Annu Rev Physiol,* 68**,** 123-58.

BIRD, S. T., HARTZEMA, A. G., BROPHY, J. M., ETMINAN, M. & DELANEY, J. A. 2013. Risk of venous thromboembolism in women with polycystic ovary syndrome: a population-based matched cohort analysis. *CMAJ,* 185**,** E115-20.

BJERSING, L. & CARSTENSEN, H. 1967. Biosynthesis of steroids by granulosa cells of the porcine ovary in vitro. *J Reprod Fertil,* 14**,** 101-11.

BJORNTORP, P. 1979. Adipose tissue morphology and morbid obesity. *Surg Clin North Am,* 59**,** 977-83.

BJORNTORP, P. 1992. Abdominal fat distribution and disease: an overview of epidemiological data. *Ann Med,* 24**,** 15-8.

BLANCHETTE, S., BLOUIN, K., RICHARD, C., DUPONT, P., LUU-THE, V. & TCHERNOF, A. 2005. Expression and activity of 20alpha-hydroxysteroid dehydrogenase (AKR1C1) in abdominal subcutaneous and omental adipose tissue in women. *The Journal of clinical endocrinology and metabolism,* 90**,** 264-70.

BLOUIN, K., BLANCHETTE, S., RICHARD, C., DUPONT, P., LUU-THE, V. & TCHERNOF, A. 2005. Expression and activity of steroid aldoketoreductases 1C in omental adipose tissue are positive correlates of adiposity in women. *American journal of physiology. Endocrinology and metabolism,* 288**,** E398-404.

BLOUIN, K., NADEAU, M., MAILLOUX, J., DARIS, M., LEBEL, S., LUU-THE, V. & TCHERNOF, A. 2009a. Pathways of adipose tissue androgen metabolism in women: depot differences and modulation by adipogenesis. *American journal of physiology. Endocrinology and metabolism,* 296**,** E244-55.

BLOUIN, K., NADEAU, M., PERREAULT, M., VEILLEUX, A., DROLET, R., MARCEAU, P., MAILLOUX, J., LUU-THE, V. & TCHERNOF, A. 2010. Effects of androgens on adipocyte differentiation and adipose tissue explant metabolism in men and women. *Clinical endocrinology,* 72**,** 176-88.

BLOUIN, K., RICHARD, C., BELANGER, C., DUPONT, P., DARIS, M., LABERGE, P., LUU-THE, V. & TCHERNOF, A. 2003. Local androgen inactivation in abdominal visceral adipose tissue. *The Journal of clinical endocrinology and metabolism,* 88**,** 5944-50.

BLOUIN, K., RICHARD, C., BROCHU, G., HOULD, F. S., LEBEL, S., MARCEAU, S., BIRON, S., LUU-THE, V. & TCHERNOF, A. 2006. Androgen inactivation and steroid-converting enzyme expression in abdominal adipose tissue in men. *The Journal of endocrinology,* 191**,** 637-49.

BLOUIN, K., VEILLEUX, A., LUU-THE, V. & TCHERNOF, A. 2009b. Androgen metabolism in adipose tissue: recent advances. *Mol Cell Endocrinol,* 301**,** 97-103.

BOLINDER, J., ENGFELDT, P., OSTMAN, J. & ARNER, P. 1983. Site differences in insulin receptor binding and insulin action in subcutaneous fat of obese females. *J Clin Endocrinol Metab,* 57**,** 455-61.

BOLT, H. M. & GOBEL, P. 1972. Formation of estrogens from androgens by human subcutaneous adipose tissue in vitro. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme,* 4**,** 312-3.

BOOTS, L. R., POTTER, S., POTTER, D. & AZZIZ, R. 1998. Measurement of total serum testosterone levels using commercially available kits: high degree of between-kit variability. *Fertil Steril,* 69**,** 286-92.

BORRUEL, S., FERNANDEZ-DURAN, E., ALPANES, M., MARTI, D., ALVAREZ-BLASCO, F., LUQUE-RAMIREZ, M. & ESCOBAR-MORREALE, H. F. 2013. Global adiposity and thickness of intraperitoneal and mesenteric adipose tissue depots are increased in women with polycystic ovary syndrome (PCOS). *J Clin Endocrinol Metab,* 98**,** 1254-63.

BRASAEMLE, D. L., RUBIN, B., HARTEN, I. A., GRUIA-GRAY, J., KIMMEL, A. R. & LONDOS, C. 2000. Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. *J Biol Chem,* 275**,** 38486-93.

BUJALSKA, I. J., KUMAR, S., HEWISON, M. & STEWART, P. M. 1999. Differentiation of adipose stromal cells: the roles of glucocorticoids and 11beta-hydroxysteroid dehydrogenase. *Endocrinology,* 140**,** 3188-96.

BUJALSKA, I. J., WALKER, E. A., TOMLINSON, J. W., HEWISON, M. & STEWART, P. M. 2002. 11Beta-hydroxysteroid dehydrogenase type 1 in differentiating omental human preadipocytes: from de-activation to generation of cortisol. *Endocr Res,* 28**,** 449-61.

BULUN, S. E. & SIMPSON, E. R. 1994. Regulation of aromatase expression in human tissues. *Breast Cancer Res Treat,* 30**,** 19-29.

BURGER, H. G. 2002. Androgen production in women. *Fertil Steril,* 77 Suppl 4**,** S3-5.

BURGHEN, G. A., GIVENS, J. R. & KITABCHI, A. E. 1980. Correlation of hyperandrogenism with hyperinsulinism in polycystic ovarian disease. *J Clin Endocrinol Metab,* 50**,** 113-6.

CALDWELL, A. S., EID, S., KAY, C. R., JIMENEZ, M., MCMAHON, A. C., DESAI, R., ALLAN, C. M., SMITH, J. T., HANDELSMAN, D. J. & WALTERS, K. A. 2015. Haplosufficient genomic androgen receptor signaling is adequate to protect female mice from induction of polycystic ovary syndrome features by prenatal hyperandrogenization. *Endocrinology***,** en20141887.

CALDWELL, A. S., MIDDLETON, L. J., JIMENEZ, M., DESAI, R., MCMAHON, A. C., ALLAN, C. M., HANDELSMAN, D. J. & WALTERS, K. A. 2014. Characterization of reproductive, metabolic, and endocrine features of polycystic ovary syndrome in female hyperandrogenic mouse models. *Endocrinology,* 155**,** 3146-59.

CAMPBELL, K. L., FOSTER-SCHUBERT, K. E., ALFANO, C. M., WANG, C. C., WANG, C. Y., DUGGAN, C. R., MASON, C., IMAYAMA, I., KONG, A., XIAO, L., BAIN, C. E., BLACKBURN, G. L., STANCZYK, F. Z. & MCTIERNAN, A. 2012. Reduced-calorie dietary weight loss, exercise, and sex hormones in postmenopausal women: randomized controlled trial. *J Clin Oncol,* 30**,** 2314-26.

CARA, J. F. & ROSENFIELD, R. L. 1988. Insulin-like growth factor I and insulin potentiate luteinizing hormone-induced androgen synthesis by rat ovarian thecal-interstitial cells. *Endocrinology,* 123**,** 733-9.

CARMINA, E. & LOBO, R. A. 2001. Polycystic ovaries in Hirsute women with normal menses. *Am J Med,* 111**,** 602-6.

CARMINA, E., ROSATO, F., JANNI, A., RIZZO, M. & LONGO, R. A. 2006. Extensive clinical experience: relative prevalence of different androgen excess disorders in 950

women referred because of clinical hyperandrogenism. *J Clin Endocrinol Metab,* 91**,** 2-6.

CARPENTIER, A., PATTERSON, B. W., LEUNG, N. & LEWIS, G. F. 2002. Sensitivity to acute insulin-mediated suppression of plasma free fatty acids is not a determinant of fasting VLDL triglyceride secretion in healthy humans. *Diabetes,* 51**,** 1867-75.

CASTERAS, A., DE SILVA, P., RUMSBY, G. & CONWAY, G. S. 2009. Reassessing fecundity in women with classical congenital adrenal hyperplasia (CAH): normal pregnancy rate but reduced fertility rate. *Clin Endocrinol (Oxf),* 70**,** 833-7.

CASTORENA, C. M., ARIAS, E. B., SHARMA, N. & CARTEE, G. D. 2014. Postexercise improvement in insulin-stimulated glucose uptake occurs concomitant with greater AS160 phosphorylation in muscle from normal and insulin-resistant rats. *Diabetes,* 63**,** 2297-308.

CAWOOD, M. L., FIELD, H. P., FORD, C. G., GILLINGWATER, S., KICMAN, A., COWAN, D. & BARTH, J. H. 2005. Testosterone measurement by isotope-dilution liquid chromatography-tandem mass spectrometry: validation of a method for routine clinical practice. *Clin Chem,* 51**,** 1472-9.

CEDERHOLM, J. & WIBELL, L. 1990. Insulin release and peripheral sensitivity at the oral glucose tolerance test. *Diabetes research and clinical practice,* 10**,** 167-75.

CHADHA, S., PACHE, T. D., HUIKESHOVEN, J. M., BRINKMANN, A. O. & VAN DER KWAST, T. H. 1994. Androgen receptor expression in human ovarian and uterine tissue of long-term androgen-treated transsexual women. *Hum Pathol,* 25**,** 1198-204.

CHADWICK, C. A., OWEN, L. J. & KEEVIL, B. G. 2005. Development of a method for the measurement of dehydroepiandrosterone sulphate by liquid chromatography-tandem mass spectrometry. *Ann Clin Biochem,* 42**,** 468-74.

CHAKHTOURA, Z., BACHELOT, A., SAMARA-BOUSTANI, D., RUIZ, J. C., DONADILLE, B., DULON, J., CHRISTIN-MAITRE, S., BOUVATTIER, C., RAUX-DEMAY, M. C., BOUCHARD, P., CAREL, J. C., LEGER, J., KUTTENN, F., POLAK, M., TOURAINE, P., CENTRE DES MALADIES ENDOCRINIENNES RARES DE LA, C. & ASSOCIATION, S. 2008. Impact of total cumulative glucocorticoid dose on bone mineral density in patients with 21-hydroxylase deficiency. *Eur J Endocrinol,* 158**,** 879-87.

CHAN, S. J., KEIM, P. & STEINER, D. F. 1976. Cell-free synthesis of rat preproinsulins: characterization and partial amino acid sequence determination. *Proc Natl Acad Sci U S A,* 73**,** 1964-8.

CHANG, R. J. & KATZ, S. E. 1999. Diagnosis of polycystic ovary syndrome. *Endocrinol Metab Clin North Am,* 28**,** 397-408, vii.

CHAZENBALK, G., CHEN, Y. H., HENEIDI, S., LEE, J. M., PALL, M., CHEN, Y. D. & AZZIZ, R. 2012. Abnormal expression of genes involved in inflammation, lipid metabolism, and Wnt signaling in the adipose tissue of polycystic ovary syndrome. *The Journal of clinical endocrinology and metabolism,* 97**,** E765-70.

CHAZENBALK, G., SINGH, P., IRGE, D., SHAH, A., ABBOTT, D. H. & DUMESIC, D. A. 2013. Androgens inhibit adipogenesis during human adipose stem cell commitment to preadipocyte formation. *Steroids,* 78**,** 920-6.

CHEATHAM, B., VLAHOS, C. J., CHEATHAM, L., WANG, L., BLENIS, J. & KAHN, C. R. 1994. Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Mol Cell Biol,* 14**,** 4902-11.

CIARALDI, T. P., ARODA, V., MUDALIAR, S., CHANG, R. J. & HENRY, R. R. 2009. Polycystic ovary syndrome is associated with tissue-specific differences in insulin resistance. *J Clin Endocrinol Metab,* 94**,** 157-63.

CIARALDI, T. P., MORALES, A. J., HICKMAN, M. G., ODOM-FORD, R., YEN, S. S. & OLEFSKY, J. M. 1998. Lack of insulin resistance in fibroblasts from subjects with polycystic ovary syndrome. *Metabolism,* 47**,** 940-6.

CLAAHSEN-VAN DER GRINTEN, H. L., OTTEN, B. J., TAKAHASHI, S., MEULEMAN, E. J., HULSBERGEN-VAN DE KAA, C., SWEEP, F. C. & HERMUS, A. R. 2007. Testicular adrenal rest tumors in adult males with congenital adrenal hyperplasia: evaluation of pituitary-gonadal function before and after successful testis-sparing surgery in eight patients. *J Clin Endocrinol Metab,* 92**,** 612-5.

CLIFFORD, G. M., MCCORMICK, D. K., VERNON, R. G. & YEAMAN, S. J. 1997. Translocation of perilipin and hormone-sensitive lipase in response to lipolytic hormones. *Biochem Soc Trans,* 25**,** S672.

COMBS, T. P., BERG, A. H., RAJALA, M. W., KLEBANOV, S., IYENGAR, P., JIMENEZ-CHILLARON, J. C., PATTI, M. E., KLEIN, S. L., WEINSTEIN, R. S. & SCHERER, P. E. 2003. Sexual differentiation, pregnancy, calorie restriction, and aging affect the adipocyte-specific secretory protein adiponectin. *Diabetes,* 52**,** 268-76.

CONSIDINE, R. V., NYCE, M. R., MORALES, L. M., MAGOSIN, S. A., SINHA, M. K., BAUER, T. L., ROSATO, E. L., COLBERG, J. & CARO, J. F. 1996. Paracrine stimulation of preadipocyte-enriched cell cultures by mature adipocytes. *Am J Physiol,* 270**,** E895-9.

CONWAY, G., DEWAILLY, D., DIAMANTI-KANDARAKIS, E., ESCOBAR-MORREALE, H. F., FRANKS, S., GAMBINERI, A., KELESTIMUR, F., MACUT, D., MICIC, D., PASQUALI, R., PFEIFER, M., PIGNATELLI, D., PUGEAT, M., YILDIZ, B. O. & GROUP, E. P. S. I. 2014. The polycystic ovary syndrome: a position statement from the European Society of Endocrinology. *Eur J Endocrinol,* 171**,** P1-29.

CORBOULD, A. 2007. Chronic testosterone treatment induces selective insulin resistance in subcutaneous adipocytes of women. *J Endocrinol,* 192**,** 585-94.

CORBOULD, A. 2008. Effects of androgens on insulin action in women: is androgen excess a component of female metabolic syndrome? *Diabetes Metab Res Rev,* 24**,** 520-32.

CORBOULD, A. M., BAWDEN, M. J., LAVRANOS, T. C., RODGERS, R. J. & JUDD, S. J. 2002. The effect of obesity on the ratio of type 3 17beta-hydroxysteroid dehydrogenase mRNA to cytochrome P450 aromatase mRNA in subcutaneous abdominal and intra-abdominal adipose tissue of women. *Int J Obes Relat Metab Disord,* 26**,** 165-75.

CORBOULD, A. M., JUDD, S. J. & RODGERS, R. J. 1998. Expression of types 1, 2, and 3 17 beta-hydroxysteroid dehydrogenase in subcutaneous abdominal and intra-abdominal adipose tissue of women. *The Journal of clinical endocrinology and metabolism,* 83**,** 187-94.

COUILLARD, C., GAGNON, J., BERGERON, J., LEON, A. S., RAO, D. C., SKINNER, J. S., WILMORE, J. H., DESPRES, J. P. & BOUCHARD, C. 2000. Contribution of body fatness and adipose tissue distribution to the age variation in plasma steroid hormone concentrations in men: the HERITAGE Family Study. *J Clin Endocrinol Metab,* 85**,** 1026-31.

CRISOSTO, N., ECHIBURU, B., MALIQUEO, M., PEREZ, V., LADRON DE GUEVARA, A., PREISLER, J., SANCHEZ, F. & SIR-PETERMANN, T. 2012. Improvement of hyperandrogenism and hyperinsulinemia during pregnancy in women with polycystic ovary syndrome: possible effect in the ovarian follicular mass of their daughters. *Fertil Steril,* 97**,** 218-24.

CUSI, K., MAEZONO, K., OSMAN, A., PENDERGRASS, M., PATTI, M. E., PRATIPANAWATR, T., DEFRONZO, R. A., KAHN, C. R. & MANDARINO, L. J. 2000. Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. *J Clin Invest,* 105**,** 311-20.

DARIMONT, C., GAILLARD, D., AILHAUD, G. & NEGREL, R. 1993. Terminal differentiation of mouse preadipocyte cells: adipogenic and antimitogenic role of triiodothyronine. *Mol Cell Endocrinol,* 98**,** 67-73.

DAVIS, S. R., MCCLOUD, P., STRAUSS, B. J. & BURGER, H. 1995. Testosterone enhances estradiol's effects on postmenopausal bone density and sexuality. *Maturitas,* 21**,** 227-36.

DAVIS, S. R. & TRAN, J. 2001. Testosterone influences libido and well being in women. *Trends Endocrinol Metab,* 12**,** 33-7.

DE GROOT, P. C., DEKKERS, O. M., ROMIJN, J. A., DIEBEN, S. W. & HELMERHORST, F. M. 2011. PCOS, coronary heart disease, stroke and the influence of obesity: a systematic review and meta-analysis. *Hum Reprod Update,* 17**,** 495-500.

DECLUE, T. J., SHAH, S. C., MARCHESE, M. & MALONE, J. I. 1991. Insulin resistance and hyperinsulinemia induce hyperandrogenism in a young type B insulin-resistant female. *J Clin Endocrinol Metab,* 72**,** 1308-11.

DESLYPERE, J. P., VERDONCK, L. & VERMEULEN, A. 1985. Fat tissue: a steroid reservoir and site of steroid metabolism. *The Journal of clinical endocrinology and metabolism,* 61**,** 564-70.

DESPRES, J. P. 2006. Intra-abdominal obesity: an untreated risk factor for Type 2 diabetes and cardiovascular disease. *Journal of endocrinological investigation,* 29**,** 77-82.

DESPRES, J. P. & LEMIEUX, I. 2006. Abdominal obesity and metabolic syndrome. *Nature,* 444**,** 881-7.

DEUGARTE, C. M., WOODS, K. S., BARTOLUCCI, A. A. & AZZIZ, R. 2006. Degree of facial and body terminal hair growth in unselected black and white women: toward a populational definition of hirsutism. *J Clin Endocrinol Metab,* 91**,** 1345-50.

DEWAILLY, D., CATTEAU-JONARD, S., REYSS, A. C., LEROY, M. & PIGNY, P. 2006. Oligoanovulation with polycystic ovaries but not overt hyperandrogenism. *The Journal of clinical endocrinology and metabolism,* 91**,** 3922-7.

DI SARRA, D., TOSI, F., BONIN, C., FIERS, T., KAUFMAN, J. M., SIGNORI, C., ZAMBOTTI, F., DALL'ALDA, M., CARUSO, B., ZANOLIN, M. E., BONORA, E. & MOGHETTI, P. 2013. Metabolic inflexibility is a feature of women with polycystic ovary syndrome and is associated with both insulin resistance and hyperandrogenism. *J Clin Endocrinol Metab,* 98**,** 2581-8.

DIAMOND, M. P., GRAINGER, D., DIAMOND, M. C., SHERWIN, R. S. & DEFRONZO, R. A. 1998. Effects of methyltestosterone on insulin secretion and sensitivity in women. *J Clin Endocrinol Metab,* 83**,** 4420-5.

DICKER, A., RYDEN, M., NASLUND, E., MUEHLEN, I. E., WIREN, M., LAFONTAN, M. & ARNER, P. 2004. Effect of testosterone on lipolysis in human pre-adipocytes from different fat depots. *Diabetologia,* 47**,** 420-8.

DIEUDONNE, M. N., PECQUERY, R., BOUMEDIENE, A., LENEVEU, M. C. & GIUDICELLI, Y. 1998. Androgen receptors in human preadipocytes and adipocytes: regional specificities and regulation by sex steroids. *The American journal of physiology,* 274**,** C1645-52.

DIEUDONNE, M. N., PECQUERY, R., LENEVEU, M. C. & GIUDICELLI, Y. 2000. Opposite effects of androgens and estrogens on adipogenesis in rat preadipocytes: evidence for sex and site-related specificities and possible involvement of insulin-like growth factor 1 receptor and peroxisome proliferator-activated receptor gamma2. *Endocrinology,* 141**,** 649-56.

DIJKSTRA, A. C., GOOS, C. M., CUNLIFFE, W. J., SULTAN, C. & VERMORKEN, A. J. 1987. Is increased 5 alpha-reductase activity a primary phenomenon in androgen-dependent skin disorders? *J Invest Dermatol,* 89**,** 87-92.

DONOHUE, W. L. & UCHIDA, I. 1954. Leprechaunism: a euphemism for a rare familial disorder. *J Pediatr,* 45**,** 505-19.

DOWMAN, J. K., HOPKINS, L. J., REYNOLDS, G. M., ARMSTRONG, M. J., NASIRI, M., NIKOLAOU, N., VAN HOUTEN, E. L., VISSER, J. A., MORGAN, S. A., LAVERY, G. G., OPRESCU, A., HUBSCHER, S. G., NEWSOME, P. N. & TOMLINSON, J. W. 2013. Loss of 5alpha-reductase type 1 accelerates the development of hepatic steatosis but protects against hepatocellular carcinoma in male mice. *Endocrinology,* 154**,** 4536-47.

DU, K., HERZIG, S., KULKARNI, R. N. & MONTMINY, M. 2003. TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. *Science,* 300**,** 1574-7.

DU, X., ROSENFIELD, R. L. & QIN, K. 2009. KLF15 Is a transcriptional regulator of the human 17beta-hydroxysteroid dehydrogenase type 5 gene. A potential link between regulation of testosterone production and fat stores in women. *J Clin Endocrinol Metab,* 94**,** 2594-601.

DUAN, J., SHAO, F., SHAO, Y., LI, J., LING, Y., TENG, K., LI, H. & WU, C. 2013. Androgen inhibits abdominal fat accumulation and negatively regulates the PCK1 gene in male chickens. *PLoS One,* 8**,** e59636.

DUFORT, I., RHEAULT, P., HUANG, X. F., SOUCY, P. & LUU-THE, V. 1999. Characteristics of a highly labile human type 5 17beta-hydroxysteroid dehydrogenase. *Endocrinology,* 140**,** 568-74.

DUMMLER, B. & HEMMINGS, B. A. 2007. Physiological roles of PKB/Akt isoforms in development and disease. *Biochem Soc Trans,* 35**,** 231-5.

DUNAIF, A. 1997. Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis. *Endocr Rev,* 18**,** 774-800.

DUNAIF, A. & GRAF, M. 1989. Insulin administration alters gonadal steroid metabolism independent of changes in gonadotropin secretion in insulin-resistant women with the polycystic ovary syndrome. *J Clin Invest,* 83**,** 23-9.

DUNAIF, A., SEGAL, K. R., FUTTERWEIT, W. & DOBRJANSKY, A. 1989. Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome. *Diabetes,* 38**,** 1165-74.

EHRMANN, D. A., BARNES, R. B. & ROSENFIELD, R. L. 1995. Polycystic ovary syndrome as a form of functional ovarian hyperandrogenism due to dysregulation of androgen secretion. *Endocrine reviews,* 16**,** 322-53.

ELBERS, J. M., DE JONG, S., TEERLINK, T., ASSCHEMAN, H., SEIDELL, J. C. & GOOREN, L. J. 1999. Changes in fat cell size and in vitro lipolytic activity of

abdominal and gluteal adipocytes after a one-year cross-sex hormone administration in transsexuals. *Metabolism,* 48**,** 1371-7.

ELBERS, J. M., GILTAY, E. J., TEERLINK, T., SCHEFFER, P. G., ASSCHEMAN, H., SEIDELL, J. C. & GOOREN, L. J. 2003. Effects of sex steroids on components of the insulin resistance syndrome in transsexual subjects. *Clin Endocrinol (Oxf),* 58**,** 562-71.

ENTENMANN, G. & HAUNER, H. 1996. Relationship between replication and differentiation in cultured human adipocyte precursor cells. *Am J Physiol,* 270**,** C1011-6.

ERICKSON, G. F., MAGOFFIN, D. A., DYER, C. A. & HOFEDITZ, C. 1985. The ovarian androgen producing cells: a review of structure/function relationships. *Endocr Rev,* 6**,** 371-99.

ERIKSEN, M. B., GLINTBORG, D., NIELSEN, M. F., JAKOBSEN, M. A., BRUSGAARD, K., TAN, Q. & GASTER, M. 2014. Testosterone treatment increases androgen receptor and aromatase gene expression in myotubes from patients with PCOS and controls, but does not induce insulin resistance. *Biochem Biophys Res Commun,* 451**,** 622-6.

ERNST, B., WILMS, B., THURNHEER, M. & SCHULTES, B. 2013. Reduced circulating androgen levels after gastric bypass surgery in severely obese women. *Obes Surg,* 23**,** 602-7.

ESCOBAR-MORREALE, H. F., ALVAREZ-BLASCO, F., BOTELLA-CARRETERO, J. I. & LUQUE-RAMIREZ, M. 2014. The striking similarities in the metabolic associations of female androgen excess and male androgen deficiency. *Hum Reprod,* 29**,** 2083-91.

ESCOBAR-MORREALE, H. F., BOTELLA-CARRETERO, J. I., ALVAREZ-BLASCO, F., SANCHO, J. & SAN MILLAN, J. L. 2005. The polycystic ovary syndrome associated with morbid obesity may resolve after weight loss induced by bariatric surgery. *J Clin Endocrinol Metab,* 90**,** 6364-9.

ESCOBAR-MORREALE, H. F., SAMINO, S., INSENSER, M., VINAIXA, M., LUQUE-RAMIREZ, M., LASUNCION, M. A. & CORREIG, X. 2012. Metabolic heterogeneity in polycystic ovary syndrome is determined by obesity: plasma metabolomic approach using GC-MS. *Clin Chem,* 58**,** 999-1009.

ESTEVE, E., RICART, W. & FERNANDEZ-REAL, J. M. 2009. Adipocytokines and insulin resistance: the possible role of lipocalin-2, retinol binding protein-4, and adiponectin. *Diabetes care,* 32 Suppl 2**,** S362-7.

EVANS, D. J., BARTH, J. H. & BURKE, C. W. 1988. Body fat topography in women with androgen excess. *Int J Obes,* 12**,** 157-62.

EZEH, U., PALL, M., MATHUR, R., DEY, D., BERMAN, D., CHEN, I. Y., DUMESIC, D. A. & AZZIZ, R. 2013. Effects of endogenous androgens and abdominal fat distribution on the interrelationship between insulin and non-insulin-mediated glucose uptake in females. *The Journal of clinical endocrinology and metabolism,* 98**,** 1541-8.

FAHLBUSCH, F. B., HEUSSNER, K., SCHMID, M., SCHILD, R., RUEBNER, M., HUEBNER, H., RASCHER, W., DOERR, H. G. & RAUH, M. 2015. Measurement of amniotic fluid steroids of midgestation via LC-MS/MS. *J Steroid Biochem Mol Biol,* 152**,** 155-160.

FAJAS, L., SCHOONJANS, K., GELMAN, L., KIM, J. B., NAJIB, J., MARTIN, G., FRUCHART, J. C., BRIGGS, M., SPIEGELMAN, B. M. & AUWERX, J. 1999. Regulation of peroxisome proliferator-activated receptor gamma expression by

adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1: implications for adipocyte differentiation and metabolism. *Mol Cell Biol,* 19**,** 5495-503.

FALCONE, T., FINEGOOD, D. T., FANTUS, I. G. & MORRIS, D. 1990. Androgen response to endogenous insulin secretion during the frequently sampled intravenous glucose tolerance test in normal and hyperandrogenic women. *J Clin Endocrinol Metab,* 71**,** 1653-7.

FALHAMMAR, H., FILIPSSON, H., HOLMDAHL, G., JANSON, P. O., NORDENSKJOLD, A., HAGENFELDT, K. & THOREN, M. 2007. Metabolic profile and body composition in adult women with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *J Clin Endocrinol Metab,* 92**,** 110-6.

FALSETTI, L., GAMBERA, A., ANDRICO, S. & SARTORI, E. 2002. Acne and hirsutism in polycystic ovary syndrome: clinical, endocrine-metabolic and ultrasonographic differences. *Gynecol Endocrinol,* 16**,** 275-84.

FARMER, S. R. 2006. Transcriptional control of adipocyte formation. *Cell Metab,* 4**,** 263-73.

FASSNACHT, M., SCHLENZ, N., SCHNEIDER, S. B., WUDY, S. A., ALLOLIO, B. & ARLT, W. 2003. Beyond adrenal and ovarian androgen generation: Increased peripheral 5 alpha-reductase activity in women with polycystic ovary syndrome. *The Journal of clinical endocrinology and metabolism,* 88**,** 2760-6.

FAUSER, B. C., TARLATZIS, B. C., REBAR, R. W., LEGRO, R. S., BALEN, A. H., LOBO, R., CARMINA, E., CHANG, J., YILDIZ, B. O., LAVEN, J. S., BOIVIN, J., PETRAGLIA, F., WIJEYERATNE, C. N., NORMAN, R. J., DUNAIF, A., FRANKS, S., WILD, R. A., DUMESIC, D. & BARNHART, K. 2012. Consensus on women's health aspects of polycystic ovary syndrome (PCOS): the Amsterdam ESHRE/ASRM-Sponsored 3rd PCOS Consensus Workshop Group. *Fertil Steril,* 97**,** 28-38 e25.

FEHER, T., HALMY, L., BODROGI, L. & KAZIK, M. H. 1976. Dehydroepiandrosterone concentration in adipose tissue of normal and overweight subjects. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme,* 8**,** 372-4.

FINKEN, M. J., ANDREWS, R. C., ANDREW, R. & WALKER, B. R. 1999. Cortisol metabolism in healthy young adults: sexual dimorphism in activities of A-ring reductases, but not 11beta-hydroxysteroid dehydrogenases. *J Clin Endocrinol Metab,* 84**,** 3316-21.

FISCHER-POSOVSZKY, P., NEWELL, F. S., WABITSCH, M. & TORNQVIST, H. E. 2008. Human SGBS cells - a unique tool for studies of human fat cell biology. *Obes Facts,* 1**,** 184-9.

FLORES-DELGADO, G., MARSCH-MORENO, M. & KURI-HARCUCH, W. 1987. Thyroid hormone stimulates adipocyte differentiation of 3T3 cells. *Mol Cell Biochem,* 76**,** 35-43.

FRANCKHAUSER, S., MUNOZ, S., PUJOL, A., CASELLAS, A., RIU, E., OTAEGUI, P., SU, B. & BOSCH, F. 2002. Increased fatty acid re-esterification by PEPCK overexpression in adipose tissue leads to obesity without insulin resistance. *Diabetes,* 51**,** 624-30.

FRANKS, S. 1989. Polycystic ovary syndrome: a changing perspective. *Clin Endocrinol (Oxf),* 31**,** 87-120.

FRANKS, S. 1995. Polycystic ovary syndrome. *N Engl J Med,* 333**,** 853-61.

FRANKS, S., GHARANI, N. & GILLING-SMITH, C. 1999. Polycystic ovary syndrome: evidence for a primary disorder of ovarian steroidogenesis. *The Journal of steroid biochemistry and molecular biology,* 69**,** 269-72.

FRANKS, S. & HARDY, K. 2010. Aberrant follicle development and anovulation in polycystic ovary syndrome. *Ann Endocrinol (Paris),* 71**,** 228-30.

FRAYN, K. N. 2010. Fat as a fuel: emerging understanding of the adipose tissue-skeletal muscle axis. *Acta Physiol (Oxf),* 199**,** 509-18.

FRIAS, J. P., MACARAEG, G. B., OFRECIO, J., YU, J. G., OLEFSKY, J. M. & KRUSZYNSKA, Y. T. 2001. Decreased susceptibility to fatty acid-induced peripheral tissue insulin resistance in women. *Diabetes,* 50**,** 1344-50.

FRUZZETTI, F., PERINI, D., LAZZARINI, V., PARRINI, D., GAMBACCIANI, M. & GENAZZANI, A. R. 2010. Comparison of effects of 3 mg drospirenone plus 20 mug ethinyl estradiol alone or combined with metformin or cyproterone acetate on classic metabolic cardiovascular risk factors in nonobese women with polycystic ovary syndrome. *Fertil Steril,* 94**,** 1793-8.

FUJIOKA, K., KAJITA, K., WU, Z., HANAMOTO, T., IKEDA, T., MORI, I., OKADA, H., YAMAUCHI, M., UNO, Y., MORITA, H., NAGANO, I., TAKAHASHI, Y. & ISHIZUKA, T. 2012. Dehydroepiandrosterone reduces preadipocyte proliferation via androgen receptor. *American journal of physiology. Endocrinology and metabolism,* 302**,** E694-704.

FUQUA, J. S. 2013. Treatment and outcomes of precocious puberty: an update. *J Clin Endocrinol Metab,* 98**,** 2198-207.

GANGER, L. K. & HAMZAVI, I. H. 2006. Excess salt and pepper hair treated with a combination of laser hair removal and topical eflornithine HCl. *J Drugs Dermatol,* 5**,** 544-5.

GAPSTUR, S. M., GANN, P. H., KOPP, P., COLANGELO, L., LONGCOPE, C. & LIU, K. 2002. Serum androgen concentrations in young men: a longitudinal analysis of associations with age, obesity, and race. The CARDIA male hormone study. *Cancer Epidemiol Biomarkers Prev,* 11**,** 1041-7.

GARAULET, M., PEREX-LLAMAS, F., FUENTE, T., ZAMORA, S. & TEBAR, F. J. 2000. Anthropometric, computed tomography and fat cell data in an obese population: relationship with insulin, leptin, tumor necrosis factor-alpha, sex hormone-binding globulin and sex hormones. *Eur J Endocrinol,* 143**,** 657-66.

GARG, A. 2011. Clinical review#: Lipodystrophies: genetic and acquired body fat disorders. *J Clin Endocrinol Metab,* 96**,** 3313-25.

GATHERCOLE, L. L., BUJALSKA, I. J., STEWART, P. M. & TOMLINSON, J. W. 2007. Glucocorticoid modulation of insulin signaling in human subcutaneous adipose tissue. *J Clin Endocrinol Metab,* 92**,** 4332-9.

GATHERCOLE, L. L., MORGAN, S. A., BUJALSKA, I. J., HAUTON, D., STEWART, P. M. & TOMLINSON, J. W. 2011. Regulation of lipogenesis by glucocorticoids and insulin in human adipose tissue. *PLoS One,* 6**,** e26223.

GESTA, S., TSENG, Y. H. & KAHN, C. R. 2007. Developmental origin of fat: tracking obesity to its source. *Cell,* 131**,** 242-56.

GILLING-SMITH, C., STORY, H., ROGERS, V. & FRANKS, S. 1997. Evidence for a primary abnormality of thecal cell steroidogenesis in the polycystic ovary syndrome. *Clinical endocrinology,* 47**,** 93-9.

GLINTBORG, D., HERMANN, A. P., HAGEN, C., JENSEN, L. T., FRYSTYK, J., BENNETT, P., FLYVBJERG, A. & ANDERSEN, M. 2009. A randomized placebo-

controlled study on the effects of pioglitazone on cortisol metabolism in polycystic ovary syndrome. *Fertil Steril,* 91**,** 842-50.

GONZALEZ, E., FLIER, E., MOLLE, D., ACCILI, D. & MCGRAW, T. E. 2011. Hyperinsulinemia leads to uncoupled insulin regulation of the GLUT4 glucose transporter and the FoxO1 transcription factor. *Proc Natl Acad Sci U S A,* 108**,** 10162-7.

GOODARZI, M. O. & AZZIZ, R. 2006. Diagnosis, epidemiology, and genetics of the polycystic ovary syndrome. *Best Pract Res Clin Endocrinol Metab,* 20**,** 193-205.

GOODARZI, M. O., ERICKSON, S., PORT, S. C., JENNRICH, R. I. & KORENMAN, S. G. 2005. beta-Cell function: a key pathological determinant in polycystic ovary syndrome. *J Clin Endocrinol Metab,* 90**,** 310-5.

GOOREN, L. J. & GILTAY, E. J. 2008. Review of studies of androgen treatment of female-to-male transsexuals: effects and risks of administration of androgens to females. *J Sex Med,* 5**,** 765-76.

GRAY, S., FEINBERG, M. W., HULL, S., KUO, C. T., WATANABE, M., SEN-BANERJEE, S., DEPINA, A., HASPEL, R. & JAIN, M. K. 2002. The Kruppel-like factor KLF15 regulates the insulin-sensitive glucose transporter GLUT4. *J Biol Chem,* 277**,** 34322-8.

GRAY, S., WANG, B., ORIHUELA, Y., HONG, E. G., FISCH, S., HALDAR, S., CLINE, G. W., KIM, J. K., PERONI, O. D., KAHN, B. B. & JAIN, M. K. 2007. Regulation of gluconeogenesis by Kruppel-like factor 15. *Cell Metab,* 5**,** 305-12.

GREENWOOD, M. R. & JOHNSON, P. R. 1977. Adipose tissue cellularity and its relationship to the development of obesity in females. *Curr Concepts Nutr,* 5**,** 119-35.

GREGSON, C. L., PAGGIOSI, M. A., CRABTREE, N., STEEL, S. A., MCCLOSKEY, E., DUNCAN, E. L., FAN, B., SHEPHERD, J. A., FRASER, W. D., SMITH, G. D. & TOBIAS, J. H. 2013. Analysis of body composition in individuals with high bone mass reveals a marked increase in fat mass in women but not men. *J Clin Endocrinol Metab,* 98**,** 818-28.

GRIMALDI, P., NEGREL, R., VINCENT, J. P. & AILHAUD, G. 1979. Differentiation of ob 17 preadipocytes to adipocytes. Effect of insulin on the levels of insulin receptors and on the transport of alpha-aminoisobutyrate. *J Biol Chem,* 254**,** 6849-52.

GRINSPOON, S. K. 2005. Metabolic syndrome and cardiovascular disease in patients with human immunodeficiency virus. *Am J Med,* 118 Suppl 2**,** 23S-28S.

GRISHKOVSKAYA, I., AVVAKUMOV, G. V., SKLENAR, G., DALES, D., HAMMOND, G. L. & MULLER, Y. A. 2000. Crystal structure of human sex hormone-binding globulin: steroid transport by a laminin G-like domain. *EMBO J,* 19**,** 504-12.

GUIDO, M., ROMUALDI, D., SURIANO, R., GIULIANI, M., COSTANTINI, B., APA, R. & LANZONE, A. 2004. Effect of pioglitazone treatment on the adrenal androgen response to corticotrophin in obese patients with polycystic ovary syndrome. *Hum Reprod,* 19**,** 534-9.

GUO, S., CICHY, S. B., HE, X., YANG, Q., RAGLAND, M., GHOSH, A. K., JOHNSON, P. F. & UNTERMAN, T. G. 2001. Insulin suppresses transactivation by CAAT/enhancer-binding proteins beta (C/EBPbeta). Signaling to p300/CREB-binding protein by protein kinase B disrupts interaction with the major activation domain of C/EBPbeta. *J Biol Chem,* 276**,** 8516-23.

GUPTA, V., BHASIN, S., GUO, W., SINGH, R., MIKI, R., CHAUHAN, P., CHOONG, K., TCHKONIA, T., LEBRASSEUR, N. K., FLANAGAN, J. N., HAMILTON, J. A., VIERECK, J. C., NARULA, N. S., KIRKLAND, J. L. & JASUJA, R. 2008. Effects of

dihydrotestosterone on differentiation and proliferation of human mesenchymal stem cells and preadipocytes. *Mol Cell Endocrinol,* 296**,** 32-40.

HAEMMERLE, G., LASS, A., ZIMMERMANN, R., GORKIEWICZ, G., MEYER, C., ROZMAN, J., HELDMAIER, G., MAIER, R., THEUSSL, C., EDER, S., KRATKY, D., WAGNER, E. F., KLINGENSPOR, M., HOEFLER, G. & ZECHNER, R. 2006. Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. *Science,* 312**,** 734-7.

HALLEUX, C. M., TAKAHASHI, M., DELPORTE, M. L., DETRY, R., FUNAHASHI, T., MATSUZAWA, Y. & BRICHARD, S. M. 2001. Secretion of adiponectin and regulation of apM1 gene expression in human visceral adipose tissue. *Biochem Biophys Res Commun,* 288**,** 1102-7.

HAMES, K. C., KOUTSARI, C., SANTOSA, S., BUSH, N. C. & JENSEN, M. D. 2015. Adipose tissue fatty acid storage factors: effects of depot, sex and fat cell size. *Int J Obes (Lond)*.

HAN, T. S., KRONE, N., WILLIS, D. S., CONWAY, G. S., HAHNER, S., REES, D. A., STIMSON, R. H., WALKER, B. R., ARLT, W., ROSS, R. J. & UNITED KINGDOM CONGENITAL ADRENAL HYPERPLASIA ADULT STUDY, E. 2013. Quality of life in adults with congenital adrenal hyperplasia relates to glucocorticoid treatment, adiposity and insulin resistance: United Kingdom Congenital adrenal Hyperplasia Adult Study Executive (CaHASE). *Eur J Endocrinol,* 168**,** 887-93.

HANSEN, F. M., FAHMY, N. & NIELSEN, J. H. 1980. The influence of sexual hormones on lipogenesis and lipolysis in rat fat cells. *Acta Endocrinol (Copenh),* 95**,** 566-70.

HANSSLER, L., SCHLOTZER, E., BLENKERS, B., ROLL, C., ZHOU, C. & KORDASS, U. 1992. [Elimination of fat emulsions of various concentrations from the blood. Observational study in the intravenous administration of Lipovenos 10% and 20% in premature infants with very low birth weight]. *Klin Padiatr,* 204**,** 27-33.

HANSSON, P., SAGGERSON, D. & NILSSON-EHLE, P. 1991. Sex difference in triglyceride/fatty acid substrate cycling of rat adipose tissue: indirect regulation by androgens. *Horm Metab Res,* 23**,** 465-8.

HAUNER, H., ENTENMANN, G., WABITSCH, M., GAILLARD, D., AILHAUD, G., NEGREL, R. & PFEIFFER, E. F. 1989. Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. *J Clin Invest,* 84**,** 1663-70.

HAUNER, H., SCHMID, P. & PFEIFFER, E. F. 1987. Glucocorticoids and insulin promote the differentiation of human adipocyte precursor cells into fat cells. *J Clin Endocrinol Metab,* 64**,** 832-5.

HAUSMAN, D. B., DIGIROLAMO, M., BARTNESS, T. J., HAUSMAN, G. J. & MARTIN, R. J. 2001. The biology of white adipocyte proliferation. *Obes Rev,* 2**,** 239-54.

HAZLEHURST, J. M., GATHERCOLE, L. L., NASIRI, M., ARMSTRONG, M. J., BORROWS, S., YU, J., WAGENMAKERS, A. J., STEWART, P. M. & TOMLINSON, J. W. 2013. Glucocorticoids fail to cause insulin resistance in human subcutaneous adipose tissue in vivo. *J Clin Endocrinol Metab,* 98**,** 1631-40.

HERMAN-GIDDENS, M. E., SLORA, E. J., WASSERMAN, R. C., BOURDONY, C. J., BHAPKAR, M. V., KOCH, G. G. & HASEMEIER, C. M. 1997. Secondary sexual characteristics and menses in young girls seen in office practice: a study from the Pediatric Research in Office Settings network. *Pediatrics,* 99**,** 505-12.

HERNANDEZ-MORANTE, J. J., PEREZ-DE-HEREDIA, F., LUJAN, J. A., ZAMORA, S. & GARAULET, M. 2008. Role of DHEA-S on body fat distribution: gender- and depot-specific stimulation of adipose tissue lipolysis. *Steroids,* 73**,** 209-15.

HILLIER, S. G. & DE ZWART, F. A. 1981. Evidence that granulosa cell aromatase induction/activation by follicle-stimulating hormone is an androgen receptor-regulated process in-vitro. *Endocrinology,* 109**,** 1303-5.

HIORT, O. 2013. The differential role of androgens in early human sex development. *BMC Med,* 11**,** 152.

HOLMANG, A., SVEDBERG, J., JENNISCHE, E. & BJORNTORP, P. 1990. Effects of testosterone on muscle insulin sensitivity and morphology in female rats. *Am J Physiol,* 259**,** E555-60.

HOLTE, J., BERGH, T., BERNE, C., BERGLUND, L. & LITHELL, H. 1994. Enhanced early insulin response to glucose in relation to insulin resistance in women with polycystic ovary syndrome and normal glucose tolerance. *J Clin Endocrinol Metab,* 78**,** 1052-8.

HORTON, R., ROMANOFF, E. & WALKER, J. 1966. Androstenedione and testosterone in ovarian venous and peripheral plasma during ovariectomy for breast cancer. *The Journal of clinical endocrinology and metabolism,* 26**,** 1267-9.

HOUSMAN, E. & REYNOLDS, R. V. 2014. Polycystic ovary syndrome: a review for dermatologists: Part I. Diagnosis and manifestations. *J Am Acad Dermatol,* 71**,** 847 e1-847 e10; quiz 857-8.

HUANG, L. Z., WANG, L., LIU, Y., XIONG, L. Q. & LI, G. F. 2011. [On-line detection of hydrocortisone concentration in rat subcutaneous adipose with liquid chromatography-mass spectrometry/mass spectrometry]. *Nan Fang Yi Ke Da Xue Xue Bao,* 31**,** 1387-91.

HUANG, W. C., LI, X., LIU, J., LIN, J. & CHUNG, L. W. 2012. Activation of androgen receptor, lipogenesis, and oxidative stress converged by SREBP-1 is responsible for regulating growth and progression of prostate cancer cells. *Mol Cancer Res,* 10**,** 133-42.

IBANEZ, L., ONG, K., DE ZEGHER, F., MARCOS, M. V., DEL RIO, L. & DUNGER, D. B. 2003. Fat distribution in non-obese girls with and without precocious pubarche: central adiposity related to insulinaemia and androgenaemia from prepuberty to postmenarche. *Clin Endocrinol (Oxf),* 58**,** 372-9.

IBANEZ, L., POTAU, N., CHACON, P., PASCUAL, C. & CARRASCOSA, A. 1998. Hyperinsulinaemia, dyslipaemia and cardiovascular risk in girls with a history of premature pubarche. *Diabetologia,* 41**,** 1057-63.

IBANEZ, L., POTAU, N., VIRDIS, R., ZAMPOLLI, M., TERZI, C., GUSSINYE, M., CARRASCOSA, A. & VICENS-CALVET, E. 1993. Postpubertal outcome in girls diagnosed of premature pubarche during childhood: increased frequency of functional ovarian hyperandrogenism. *J Clin Endocrinol Metab,* 76**,** 1599-603.

IBANEZ, L., POTAU, N., ZAMPOLLI, M., PRAT, N., VIRDIS, R., VICENS-CALVET, E. & CARRASCOSA, A. 1996. Hyperinsulinemia in postpubertal girls with a history of premature pubarche and functional ovarian hyperandrogenism. *J Clin Endocrinol Metab,* 81**,** 1237-43.

IBANEZ, L., POTAU, N., ZAMPOLLI, M., RIQUE, S., SAENGER, P. & CARRASCOSA, A. 1997. Hyperinsulinemia and decreased insulin-like growth factor-binding protein-1 are common features in prepubertal and pubertal girls with a history of premature pubarche. *J Clin Endocrinol Metab,* 82**,** 2283-8.

IDKOWIAK, J., LAVERY, G. G., DHIR, V., BARRETT, T. G., STEWART, P. M., KRONE, N. & ARLT, W. 2011. Premature adrenarche: novel lessons from early onset androgen excess. *European journal of endocrinology / European Federation of Endocrine Societies,* 165**,** 189-207.

ITANI, S. I., SAHA, A. K., KUROWSKI, T. G., COFFIN, H. R., TORNHEIM, K. & RUDERMAN, N. B. 2003. Glucose autoregulates its uptake in skeletal muscle: involvement of AMP-activated protein kinase. *Diabetes,* 52**,** 1635-40.

JAKUBOWICZ, D. J. & NESTLER, J. E. 1997. 17 alpha-Hydroxyprogesterone responses to leuprolide and serum androgens in obese women with and without polycystic ovary syndrome offer dietary weight loss. *J Clin Endocrinol Metab,* 82**,** 556-60.

JAMDAR, S. C. 1978. Glycerolipid biosynthesis in rat adipose tissue. Influence of adipose-cell size and site of adipose tissue on triacylglycerol formation in lean and obese rats. *Biochem J,* 170**,** 153-60.

JAYASENA, C. N. & FRANKS, S. 2014. The management of patients with polycystic ovary syndrome. *Nat Rev Endocrinol,* 10**,** 624-36.

JENSTERLE, M., KOCJAN, T. & JANEZ, A. 2014. Phosphodiesterase 4 inhibition as a potential new therapeutic target in obese women with polycystic ovary syndrome. *J Clin Endocrinol Metab,* 99**,** E1476-81.

JOINT, L. E. C. A. H. W. G. 2002. Consensus statement on 21-hydroxylase deficiency from the Lawson Wilkins Pediatric Endocrine Society and the European Society for Paediatric Endocrinology. *J Clin Endocrinol Metab,* 87**,** 4048-53.

JONES, H., SPRUNG, V. S., PUGH, C. J., DAOUSI, C., IRWIN, A., AZIZ, N., ADAMS, V. L., THOMAS, E. L., BELL, J. D., KEMP, G. J. & CUTHBERTSON, D. J. 2012. Polycystic ovary syndrome with hyperandrogenism is characterized by an increased risk of hepatic steatosis compared to nonhyperandrogenic PCOS phenotypes and healthy controls, independent of obesity and insulin resistance. *J Clin Endocrinol Metab,* 97**,** 3709-16.

JONES, M. E., SCHOEMAKER, M., RAE, M., FOLKERD, E. J., DOWSETT, M., ASHWORTH, A. & SWERDLOW, A. J. 2013. Changes in estradiol and testosterone levels in postmenopausal women after changes in body mass index. *J Clin Endocrinol Metab,* 98**,** 2967-74.

JOYNER, J., HUTLEY, L. & CAMERON, D. 2002. Intrinsic regional differences in androgen receptors and dihydrotestosterone metabolism in human preadipocytes. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme,* 34**,** 223-8.

JU, R., WU, W., FEI, J., QIN, Y., TANG, Q., WU, D., XIA, Y., WU, J. & WANG, X. 2015. Association analysis between the polymorphisms of HSD17B5 and HSD17B6 and risk of polycystic ovary syndrome in Chinese population. *Eur J Endocrinol,* 172**,** 227-33.

KAMRATH, C., HARTMANN, M. F. & WUDY, S. A. 2013. Androgen synthesis in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Horm Metab Res,* 45**,** 86-91.

KANSARA, M. S., MEHRA, A. K., VON HAGEN, J., KABOTYANSKY, E. & SMITH, P. J. 1996. Physiological concentrations of insulin and T3 stimulate 3T3-L1 adipocyte acyl-CoA synthetase gene transcription. *Am J Physiol,* 270**,** E873-81.

KAPUR, S. P. & REDDI, A. H. 1989. Influence of testosterone and dihydrotestosterone on bone-matrix induced endochondral bone formation. *Calcif Tissue Int,* 44**,** 108-13.

KARBOWSKA, J. & KOCHAN, Z. 2012. Fat-reducing effects of dehydroepiandrosterone involve upregulation of ATGL and HSL expression, and stimulation of lipolysis in adipose tissue. *Steroids,* 77**,** 1359-65.

KASPERK, C. H., WERGEDAL, J. E., FARLEY, J. R., LINKHART, T. A., TURNER, R. T. & BAYLINK, D. J. 1989. Androgens directly stimulate proliferation of bone cells in vitro. *Endocrinology,* 124**,** 1576-8.

KEMPNA, P., HOFER, G., MULLIS, P. E. & FLUCK, C. E. 2007. Pioglitazone inhibits androgen production in NCI-H295R cells by regulating gene expression of CYP17 and HSD3B2. *Mol Pharmacol,* 71**,** 787-98.

KERSHAW, E. E. & FLIER, J. S. 2004. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab,* 89**,** 2548-56.

KETEL, I. J., STEHOUWER, C. D., HENRY, R. M., SERNE, E. H., HOMPES, P., HOMBURG, R., SMULDERS, Y. M. & LAMBALK, C. B. 2010. Greater arterial stiffness in polycystic ovary syndrome (PCOS) is an obesity--but not a PCOS-associated phenomenon. *J Clin Endocrinol Metab,* 95**,** 4566-75.

KIM, Y. B., SHULMAN, G. I. & KAHN, B. B. 2002. Fatty acid infusion selectively impairs insulin action on Akt1 and protein kinase C lambda /zeta but not on glycogen synthase kinase-3. *J Biol Chem,* 277**,** 32915-22.

KINOSHITA, T., HONMA, S., SHIBATA, Y., YAMASHITA, K., WATANABE, Y., MAEKUBO, H., OKUYAMA, M., TAKASHIMA, A. & TAKESHITA, N. 2014. An innovative LC-MS/MS-based method for determining CYP 17 and CYP 19 activity in the adipose tissue of pre- and postmenopausal and ovariectomized women using C-labeled steroid substrates. *J Clin Endocrinol Metab,* jc20133715.

KNOBLER, H. & ELSON, A. 2014. Metabolic regulation by protein tyrosine phosphatases. *J Biomed Res,* 28**,** 157-68.

KNOCHENHAUER, E. S., KEY, T. J., KAHSAR-MILLER, M., WAGGONER, W., BOOTS, L. R. & AZZIZ, R. 1998. Prevalence of the polycystic ovary syndrome in unselected black and white women of the southeastern United States: a prospective study. *The Journal of clinical endocrinology and metabolism,* 83**,** 3078-82.

KOLIAKI, C. & RODEN, M. 2013. Hepatic energy metabolism in human diabetes mellitus, obesity and non-alcoholic fatty liver disease. *Mol Cell Endocrinol,* 379**,** 35-42.

KOPP, H. P., KRZYZANOWSKA, K., SCHERNTHANER, G. H., KRIWANEK, S. & SCHERNTHANER, G. 2006. Relationship of androgens to insulin resistance and chronic inflammation in morbidly obese premenopausal women: studies before and after vertical banded gastroplasty. *Obes Surg,* 16**,** 1214-20.

KRANENDONK, M. E., VAN HERWAARDEN, J. A., STUPKOVA, T., JAGER, W., VINK, A., MOLL, F. L., KALKHOVEN, E. & VISSEREN, F. L. 2015. Inflammatory characteristics of distinct abdominal adipose tissue depots relate differently to metabolic risk factors for cardiovascular disease: Distinct fat depots and vascular risk factors. *Atherosclerosis,* 239**,** 419-427.

KUBOTA, N., TERAUCHI, Y., MIKI, H., TAMEMOTO, H., YAMAUCHI, T., KOMEDA, K., SATOH, S., NAKANO, R., ISHII, C., SUGIYAMA, T., ETO, K., TSUBAMOTO, Y., OKUNO, A., MURAKAMI, K., SEKIHARA, H., HASEGAWA, G., NAITO, M., TOYOSHIMA, Y., TANAKA, S., SHIOTA, K., KITAMURA, T., FUJITA, T., EZAKI, O., AIZAWA, S., KADOWAKI, T. & ET AL. 1999. PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol Cell,* 4**,** 597-609.

KUMAR, A., WOODS, K. S., BARTOLUCCI, A. A. & AZZIZ, R. 2005. Prevalence of adrenal androgen excess in patients with the polycystic ovary syndrome (PCOS). *Clinical endocrinology,* 62**,** 644-9.

KUSHNIR, M. M., BLAMIRES, T., ROCKWOOD, A. L., ROBERTS, W. L., YUE, B., ERDOGAN, E., BUNKER, A. M. & MEIKLE, A. W. 2010. Liquid chromatography-tandem mass spectrometry assay for androstenedione, dehydroepiandrosterone, and testosterone with pediatric and adult reference intervals. *Clin Chem,* 56**,** 1138-47.

L'ALLEMAND, D., PENHOAT, A., LEBRETHON, M. C., ARDEVOL, R., BAEHR, V., OELKERS, W. & SAEZ, J. M. 1996. Insulin-like growth factors enhance steroidogenic enzyme and corticotropin receptor messenger ribonucleic acid levels and corticotropin steroidogenic responsiveness in cultured human adrenocortical cells. *J Clin Endocrinol Metab,* 81**,** 3892-7.

LABRIE, F. 1991. Intracrinology. *Mol Cell Endocrinol,* 78**,** C113-8.

LABRIE, F., BELANGER, A., CUSAN, L. & CANDAS, B. 1997a. Physiological changes in dehydroepiandrosterone are not reflected by serum levels of active androgens and estrogens but of their metabolites: intracrinology. *J Clin Endocrinol Metab,* 82**,** 2403-9.

LABRIE, F., BELANGER, A., CUSAN, L., GOMEZ, J. L. & CANDAS, B. 1997b. Marked decline in serum concentrations of adrenal C19 sex steroid precursors and conjugated androgen metabolites during aging. *J Clin Endocrinol Metab,* 82**,** 2396-402.

LABRIE, F., LUU-THE, V., LABRIE, C., BELANGER, A., SIMARD, J., LIN, S. X. & PELLETIER, G. 2003. Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone. *Endocr Rev,* 24**,** 152-82.

LACASA, D., AGLI, B., MOYNARD, D. & GIUDICELLI, Y. 1995. Evidence for a regional-specific control of rat preadipocyte proliferation and differentiation by the androgenic status. *Endocrine,* 3**,** 789-93.

LACHELIN, G. C., JUDD, H. L., SWANSON, S. C., HAUCK, M. E., PARKER, D. C. & YEN, S. S. 1982. Long term effects of nightly dexamethasone administration in patients with polycystic ovarian disease. *J Clin Endocrinol Metab,* 55**,** 768-73.

LANFRANCO, F., ZITZMANN, M., SIMONI, M. & NIESCHLAG, E. 2004. Serum adiponectin levels in hypogonadal males: influence of testosterone replacement therapy. *Clinical endocrinology,* 60**,** 500-7.

LAVEN, J. S., MULDERS, A. G., VISSER, J. A., THEMMEN, A. P., DE JONG, F. H. & FAUSER, B. C. 2004. Anti-Mullerian hormone serum concentrations in normoovulatory and anovulatory women of reproductive age. *J Clin Endocrinol Metab,* 89**,** 318-23.

LAYDEN, B. T., DURAI, V., NEWMAN, M. V., MARINELARENA, A. M., AHN, C. W., FENG, G., LIN, S., ZHANG, X., KAUFMAN, D. B., JAFARI, N., SORENSEN, G. L. & LOWE, W. L., JR. 2010. Regulation of pancreatic islet gene expression in mouse islets by pregnancy. *J Endocrinol,* 207**,** 265-79.

LEA-CURRIE, Y. R., WEN, P. & MCINTOSH, M. K. 1998. Dehydroepiandrosterone reduces proliferation and differentiation of 3T3-L1 preadipocytes. *Biochem Biophys Res Commun,* 248**,** 497-504.

LEBBE, M. & WOODRUFF, T. K. 2013. Involvement of androgens in ovarian health and disease. *Mol Hum Reprod,* 19**,** 828-37.

LEE, J. & PILCH, P. F. 1994. The insulin receptor: structure, function, and signaling. *Am J Physiol,* 266**,** C319-34.

LEGRO, R. S., BARNHART, H. X., SCHLAFF, W. D., CARR, B. R., DIAMOND, M. P., CARSON, S. A., STEINKAMPF, M. P., COUTIFARIS, C., MCGOVERN, P. G., CATALDO, N. A., GOSMAN, G. G., NESTLER, J. E., GIUDICE, L. C., LEPPERT, P. C., MYERS, E. R. & COOPERATIVE MULTICENTER REPRODUCTIVE MEDICINE, N. 2007. Clomiphene, metformin, or both for infertility in the polycystic ovary syndrome. *N Engl J Med,* 356**,** 551-66.

LEGRO, R. S., BENTLEY-LEWIS, R., DRISCOLL, D., WANG, S. C. & DUNAIF, A. 2002. Insulin resistance in the sisters of women with polycystic ovary syndrome: association with hyperandrogenemia rather than menstrual irregularity. *J Clin Endocrinol Metab,* 87**,** 2128-33.

LEGRO, R. S., BRZYSKI, R. G., DIAMOND, M. P., COUTIFARIS, C., SCHLAFF, W. D., CASSON, P., CHRISTMAN, G. M., HUANG, H., YAN, Q., ALVERO, R., HAISENLEDER, D. J., BARNHART, K. T., BATES, G. W., USADI, R., LUCIDI, S., BAKER, V., TRUSSELL, J. C., KRAWETZ, S. A., SNYDER, P., OHL, D., SANTORO, N., EISENBERG, E., ZHANG, H. & NETWORK, N. R. M. 2014. Letrozole versus clomiphene for infertility in the polycystic ovary syndrome. *N Engl J Med,* 371**,** 119-29.

LEGRO, R. S., KUNSELMAN, A. R., DODSON, W. C. & DUNAIF, A. 1999. Prevalence and predictors of risk for type 2 diabetes mellitus and impaired glucose tolerance in polycystic ovary syndrome: a prospective, controlled study in 254 affected women. *J Clin Endocrinol Metab,* 84**,** 165-9.

LEMIEUX, S., PRUD'HOMME, D., BOUCHARD, C., TREMBLAY, A. & DESPRES, J. P. 1993. Sex differences in the relation of visceral adipose tissue accumulation to total body fatness. *Am J Clin Nutr,* 58**,** 463-7.

LI, Y., HE, P. P., ZHANG, D. W., ZHENG, X. L., CAYABYAB, F. S., YIN, W. D. & TANG, C. K. 2014. Lipoprotein lipase: from gene to atherosclerosis. *Atherosclerosis,* 237**,** 597-608.

LIDEGAARD, O., NIELSEN, L. H., SKOVLUND, C. W., SKJELDESTAD, F. E. & LOKKEGAARD, E. 2011. Risk of venous thromboembolism from use of oral contraceptives containing different progestogens and oestrogen doses: Danish cohort study, 2001-9. *BMJ,* 343**,** d6423.

LIEDTKE, A. J., ADENIJI, A. O., CHEN, M., BYRNS, M. C., JIN, Y., CHRISTIANSON, D. W., MARNETT, L. J. & PENNING, T. M. 2013. Development of potent and selective indomethacin analogues for the inhibition of AKR1C3 (Type 5 17beta-hydroxysteroid dehydrogenase/prostaglandin F synthase) in castrate-resistant prostate cancer. *J Med Chem,* 56**,** 2429-46.

LISCH, H. J., SAILER, S., SANDHOFER, F., SCHENNACH, W. & BRAUNSTEINER, H. 1973. Action of insulin and glucose on the re-esterification rate of free fatty acids in isolated human fat cells. *Diabetologia,* 9**,** 499-504.

LIU, F., KIM, J., LI, Y., LIU, X., LI, J. & CHEN, X. 2001. An extract of Lagerstroemia speciosa L. has insulin-like glucose uptake-stimulatory and adipocyte differentiation-inhibitory activities in 3T3-L1 cells. *J Nutr,* 131**,** 2242-7.

LOGAN, K. A., JUENGEL, J. L. & MCNATTY, K. P. 2002. Onset of steroidogenic enzyme gene expression during ovarian follicular development in sheep. *Biol Reprod,* 66**,** 906-16.

LONGCOPE, C. 1986. Adrenal and gonadal androgen secretion in normal females. *Clin Endocrinol Metab,* 15**,** 213-28.

LOUGHLIN, T., CUNNINGHAM, S., MOORE, A., CULLITON, M., SMYTH, P. P. & MCKENNA, T. J. 1986. Adrenal abnormalities in polycystic ovary syndrome. *The Journal of clinical endocrinology and metabolism,* 62**,** 142-7.

LUU-THE, V., TAKAHASHI, M., DE LAUNOIT, Y., DUMONT, M., LACHANCE, Y. & LABRIE, F. 1991. Evidence for distinct dehydrogenase and isomerase sites within a single 3 beta-hydroxysteroid dehydrogenase/5-ene-4-ene isomerase protein. *Biochemistry,* 30**,** 8861-5.

MACKENZIE, S. M., HUDA, S. S., SATTAR, N., FRASER, R., CONNELL, J. M. & DAVIES, E. 2008. Depot-specific steroidogenic gene transcription in human adipose tissue. *Clin Endocrinol (Oxf),* 69**,** 848-54.

MAGOFFIN, D. A. 2002. The ovarian androgen-producing cells: a 2001 perspective. *Rev Endocr Metab Disord,* 3**,** 47-53.

MAGOFFIN, D. A. 2005. Ovarian theca cell. *Int J Biochem Cell Biol,* 37**,** 1344-9.

MANI, H., LEVY, M. J., DAVIES, M. J., MORRIS, D. H., GRAY, L. J., BANKART, J., BLACKLEDGE, H., KHUNTI, K. & HOWLETT, T. A. 2013. Diabetes and cardiovascular events in women with polycystic ovary syndrome: a 20-year retrospective cohort study. *Clin Endocrinol (Oxf),* 78**,** 926-34.

MANNERAS-HOLM, L., LEONHARDT, H., KULLBERG, J., JENNISCHE, E., ODEN, A., HOLM, G., HELLSTROM, M., LONN, L., OLIVECRONA, G., STENER-VICTORIN, E. & LONN, M. 2011. Adipose tissue has aberrant morphology and function in PCOS: enlarged adipocytes and low serum adiponectin, but not circulating sex steroids, are strongly associated with insulin resistance. *J Clin Endocrinol Metab,* 96**,** E304-11.

MARIN, P., LONN, L., ANDERSSON, B., ODEN, B., OLBE, L., BENGTSSON, B. A. & BJORNTORP, P. 1996. Assimilation of triglycerides in subcutaneous and intraabdominal adipose tissues in vivo in men: effects of testosterone. *The Journal of clinical endocrinology and metabolism,* 81**,** 1018-22.

MARTINEZ-GARCIA, M. A., MONTES-NIETO, R., FERNANDEZ-DURAN, E., INSENSER, M., LUQUE-RAMIREZ, M. & ESCOBAR-MORREALE, H. F. 2013. Evidence for masculinization of adipokine gene expression in visceral and subcutaneous adipose tissue of obese women with polycystic ovary syndrome (PCOS). *The Journal of clinical endocrinology and metabolism,* 98**,** E388-96.

MASOODI, M., KUDA, O., ROSSMEISL, M., FLACHS, P. & KOPECKY, J. 2015. Lipid signaling in adipose tissue: Connecting inflammation & metabolism. *Biochim Biophys Acta,* 1851**,** 503-518.

MATSUBARA, M., MARUOKA, S. & KATAYOSE, S. 2002. Decreased plasma adiponectin concentrations in women with dyslipidemia. *The Journal of clinical endocrinology and metabolism,* 87**,** 2764-9.

MAYES, P. A. 1970. Studies on the major pathways of hepatic lipid metabolism using the perfused liver. *Horm Metab Res,* 2**,** Suppl 2:186-95.

MCGEE, E., SAWETAWAN, C., BIRD, I., RAINEY, W. E. & CARR, B. R. 1995. The effects of insulin on 3 beta-hydroxysteroid dehydrogenase expression in human luteinized granulosa cells. *J Soc Gynecol Investig,* 2**,** 535-41.

MCGEE, E. A., SAWETAWAN, C., BIRD, I., RAINEY, W. E. & CARR, B. R. 1996. The effect of insulin and insulin-like growth factors on the expression of steroidogenic enzymes in a human ovarian thecal-like tumor cell model. *Fertil Steril,* 65**,** 87-93.

MCNELIS, J. C., MANOLOPOULOS, K. N., GATHERCOLE, L. L., BUJALSKA, I. J., STEWART, P. M., TOMLINSON, J. W. & ARLT, W. 2013. Dehydroepiandrosterone

exerts anti-glucocorticoid action on human preadipocyte proliferation, differentiation and glucose uptake. *American journal of physiology. Endocrinology and metabolism*.

MCNICOL, A. M. 2008. A diagnostic approach to adrenal cortical lesions. *Endocr Pathol,* 19**,** 241-51.

MENDEZ, E., MONTSERRAT, N. & PLANAS, J. V. 2005. Modulation of the steroidogenic activity of luteinizing hormone by insulin and insulin-like growth factor-I through interaction with the cAMP-dependent protein kinase signaling pathway in the trout ovary. *Mol Cell Endocrinol,* 229**,** 49-56.

MENTING, J. G., YANG, Y., CHAN, S. J., PHILLIPS, N. B., SMITH, B. J., WHITTAKER, J., WICKRAMASINGHE, N. P., WHITTAKER, L. J., PANDYARAJAN, V., WAN, Z. L., YADAV, S. P., CARROLL, J. M., STROKES, N., ROBERTS, C. T., JR., ISMAIL-BEIGI, F., MILEWSKI, W., STEINER, D. F., CHAUHAN, V. S., WARD, C. W., WEISS, M. A. & LAWRENCE, M. C. 2014. Protective hinge in insulin opens to enable its receptor engagement. *Proc Natl Acad Sci U S A,* 111**,** E3395-404.

MERZ, C. N., JOHNSON, B. D., BERGA, S., BRAUNSTEIN, G., REIS, S. E., BITTNER, V. & GROUP, W. S. 2006. Past oral contraceptive use and angiographic coronary artery disease in postmenopausal women: data from the National Heart, Lung, and Blood Institute-sponsored Women's Ischemia Syndrome Evaluation. *Fertil Steril,* 85**,** 1425-31.

METHLIE, P., DANKEL, S., MYHRA, T., CHRISTENSEN, B., GJERDE, J., FADNES, D., VAGE, V., LOVAS, K. & MELLGREN, G. 2013. Changes in adipose glucocorticoid metabolism before and after bariatric surgery assessed by direct hormone measurements. *Obesity (Silver Spring),* 21**,** 2495-503.

MILEWICH, L., SHAW, C. E., MASON, J. I., CARR, B. R., BLOMQUIST, C. H. & THOMAS, J. L. 1993. 3 beta-hydroxysteroid dehydrogenase activity in tissues of the human fetus determined with 5 alpha-androstane-3 beta,17 beta-diol and dehydroepiandrosterone as substrates. *J Steroid Biochem Mol Biol,* 45**,** 525-37.

MISRA, A., PEETHAMBARAM, A. & GARG, A. 2004. Clinical features and metabolic and autoimmune derangements in acquired partial lipodystrophy: report of 35 cases and review of the literature. *Medicine (Baltimore),* 83**,** 18-34.

MIYAKE, K., OGAWA, W., MATSUMOTO, M., NAKAMURA, T., SAKAUE, H. & KASUGA, M. 2002. Hyperinsulinemia, glucose intolerance, and dyslipidemia induced by acute inhibition of phosphoinositide 3-kinase signaling in the liver. *J Clin Invest,* 110**,** 1483-91.

MIYAZAKI, J., ARAKI, K., YAMATO, E., IKEGAMI, H., ASANO, T., SHIBASAKI, Y., OKA, Y. & YAMAMURA, K. 1990. Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology,* 127**,** 126-32.

MOGHETTI, P., CASTELLO, R., NEGRI, C., TOSI, F., SPIAZZI, G. G., BRUN, E., BALDUCCI, R., TOSCANO, V. & MUGGEO, M. 1996. Insulin infusion amplifies 17 alpha-hydroxycorticosteroid intermediates response to adrenocorticotropin in hyperandrogenic women: apparent relative impairment of 17,20-lyase activity. *J Clin Endocrinol Metab,* 81**,** 881-6.

MOGHETTI, P., TOSI, F., BONIN, C., DI SARRA, D., FIERS, T., KAUFMAN, J. M., GIAGULLI, V. A., SIGNORI, C., ZAMBOTTI, F., DALL'ALDA, M., SPIAZZI, G., ZANOLIN, M. E. & BONORA, E. 2013. Divergences in insulin resistance between the different phenotypes of the polycystic ovary syndrome. *J Clin Endocrinol Metab,* 98**,** E628-37.

MOGHETTI, P., TOSI, F., TOSTI, A., NEGRI, C., MISCIALI, C., PERRONE, F., CAPUTO, M., MUGGEO, M. & CASTELLO, R. 2000. Comparison of spironolactone, flutamide, and finasteride efficacy in the treatment of hirsutism: a randomized, double blind, placebo-controlled trial. *J Clin Endocrinol Metab,* 85**,** 89-94.

MONGRAW-CHAFFIN, M. L., ANDERSON, C. A., ALLISON, M. A., OUYANG, P., SZKLO, M., VAIDYA, D., WOODWARD, M. & GOLDEN, S. H. 2015. Association between sex hormones and adiposity: qualitative differences in women and men in the multi-ethnic study of atherosclerosis. *J Clin Endocrinol Metab,* 100**,** E596-600.

MONTES-NIETO, R., INSENSER, M., MARTINEZ-GARCIA, M. A. & ESCOBAR-MORREALE, H. F. 2013. A nontargeted proteomic study of the influence of androgen excess on human visceral and subcutaneous adipose tissue proteomes. *J Clin Endocrinol Metab,* 98**,** E576-85.

MONTGOMERY, J. S., PRICE, D. K. & FIGG, W. D. 2001. The androgen receptor gene and its influence on the development and progression of prostate cancer. *J Pathol,* 195**,** 138-46.

MORI, T., SAKAUE, H., IGUCHI, H., GOMI, H., OKADA, Y., TAKASHIMA, Y., NAKAMURA, K., NAKAMURA, T., YAMAUCHI, T., KUBOTA, N., KADOWAKI, T., MATSUKI, Y., OGAWA, W., HIRAMATSU, R. & KASUGA, M. 2005. Role of Kruppel-like factor 15 (KLF15) in transcriptional regulation of adipogenesis. *J Biol Chem,* 280**,** 12867-75.

MORIMOTO, S., FERNANDEZ-MEJIA, C., ROMERO-NAVARRO, G., MORALES-PEZA, N. & DIAZ-SANCHEZ, V. 2001. Testosterone effect on insulin content, messenger ribonucleic acid levels, promoter activity, and secretion in the rat. *Endocrinology,* 142**,** 1442-7.

MORO, C., PASARICA, M., ELKIND-HIRSCH, K. & REDMAN, L. M. 2009. Aerobic exercise training improves atrial natriuretic peptide and catecholamine-mediated lipolysis in obese women with polycystic ovary syndrome. *J Clin Endocrinol Metab,* 94**,** 2579-86.

MORTON, A. 2013. Neutrophil leucocytosis and polycystic ovarian syndrome--could elevated noradrenaline levels due to obstructive sleep apnoea be a contributing mechanism? *Clin Endocrinol (Oxf),* 79**,** 591.

MOURITSEN, A., JUUL, A. & JORGENSEN, N. 2010. Improvement of semen quality in an infertile man with 21-hydroxylase deficiency, suppressed serum gonadotropins and testicular adrenal rest tumours. *Int J Androl,* 33**,** 518-20.

MOUSTAID, N., JONES, B. H. & TAYLOR, J. W. 1996. Insulin increases lipogenic enzyme activity in human adipocytes in primary culture. *J Nutr,* 126**,** 865-70.

MUNZKER, J., HOFER, D., TRUMMER, C., ULBING, M., HARGER, A., PIEBER, T., OWEN, L., KEEVIL, B., BRABANT, G., LERCHBAUM, E. & OBERMAYER-PIETSCH, B. 2015. Testosterone to dihydrotestosterone ratio as a new biomarker for an adverse metabolic phenotype in the polycystic ovary syndrome. *J Clin Endocrinol Metab,* 100**,** 653-60.

MUSSO, C., COCHRAN, E., MORAN, S. A., SKARULIS, M. C., ORAL, E. A., TAYLOR, S. & GORDEN, P. 2004. Clinical course of genetic diseases of the insulin receptor (type A and Rabson-Mendenhall syndromes): a 30-year prospective. *Medicine (Baltimore),* 83**,** 209-22.

MUSSO, C., SHAWKER, T., COCHRAN, E., JAVOR, E. D., YOUNG, J. & GORDEN, P. 2005. Clinical evidence that hyperinsulinaemia independent of gonadotropins stimulates ovarian growth. *Clin Endocrinol (Oxf),* 63**,** 73-8.

NADER, S. & DIAMANTI-KANDARAKIS, E. 2007. Polycystic ovary syndrome, oral contraceptives and metabolic issues: new perspectives and a unifying hypothesis. *Hum Reprod,* 22**,** 317-22.

NAKAMURA, Y., HORNSBY, P. J., CASSON, P., MORIMOTO, R., SATOH, F., XING, Y., KENNEDY, M. R., SASANO, H. & RAINEY, W. E. 2009. Type 5 17beta-hydroxysteroid dehydrogenase (AKR1C3) contributes to testosterone production in the adrenal reticularis. *J Clin Endocrinol Metab,* 94**,** 2192-8.

NASIRI, M., NIKOLAOU, N., PARAJES, S., KRONE, N. P., VALSAMAKIS, G., MASTORAKOS, G., HUGHES, B., TAYLOR, A., BUJALSKA, I. J., GATHERCOLE, L. L. & TOMLINSON, J. W. 2015. 5alpha-reductase type 2 regulates glucocorticoid action and metabolic phenotype in human hepatocytes. *Endocrinology***,** en20151149.

NEDERGAARD, J., BENGTSSON, T. & CANNON, B. 2007. Unexpected evidence for active brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab,* 293**,** E444-52.

NEDERGAARD, J. & CANNON, B. 2013. How brown is brown fat? It depends where you look. *Nat Med,* 19**,** 540-1.

NEDUNGADI, T. P. & CLEGG, D. J. 2009. Sexual dimorphism in body fat distribution and risk for cardiovascular diseases. *J Cardiovasc Transl Res,* 2**,** 321-7.

NEELY, E. K. & CROSSEN, S. S. 2014. Precocious puberty. *Curr Opin Obstet Gynecol,* 26**,** 332-8.

NELSON, V. L., QIN, K. N., ROSENFIELD, R. L., WOOD, J. R., PENNING, T. M., LEGRO, R. S., STRAUSS, J. F., 3RD & MCALLISTER, J. M. 2001. The biochemical basis for increased testosterone production in theca cells propagated from patients with polycystic ovary syndrome. *J Clin Endocrinol Metab,* 86**,** 5925-33.

NESTLER, J. E. & JAKUBOWICZ, D. J. 1996. Decreases in ovarian cytochrome P450c17 alpha activity and serum free testosterone after reduction of insulin secretion in polycystic ovary syndrome. *N Engl J Med,* 335**,** 617-23.

NESTLER, J. E. & JAKUBOWICZ, D. J. 1997. Lean women with polycystic ovary syndrome respond to insulin reduction with decreases in ovarian P450c17 alpha activity and serum androgens. *J Clin Endocrinol Metab,* 82**,** 4075-9.

NESTLER, J. E., JAKUBOWICZ, D. J., DE VARGAS, A. F., BRIK, C., QUINTERO, N. & MEDINA, F. 1998. Insulin stimulates testosterone biosynthesis by human thecal cells from women with polycystic ovary syndrome by activating its own receptor and using inositolglycan mediators as the signal transduction system. *J Clin Endocrinol Metab,* 83**,** 2001-5.

NISHIZAWA, H., SHIMOMURA, I., KISHIDA, K., MAEDA, N., KURIYAMA, H., NAGARETANI, H., MATSUDA, M., KONDO, H., FURUYAMA, N., KIHARA, S., NAKAMURA, T., TOCHINO, Y., FUNAHASHI, T. & MATSUZAWA, Y. 2002. Androgens decrease plasma adiponectin, an insulin-sensitizing adipocyte-derived protein. *Diabetes,* 51**,** 2734-41.

NOHARA, K., LAQUE, A., ALLARD, C., MUNZBERG, H. & MAUVAIS-JARVIS, F. 2014. Central mechanisms of adiposity in adult female mice with androgen excess. *Obesity (Silver Spring),* 22**,** 1477-84.

NOORDAM, C., DHIR, V., MCNELIS, J. C., SCHLERETH, F., HANLEY, N. A., KRONE, N., SMEITINK, J. A., SMEETS, R., SWEEP, F. C., CLAAHSEN-VAN DER GRINTEN, H. L. & ARLT, W. 2009. Inactivating PAPSS2 mutations in a patient with premature pubarche. *The New England journal of medicine,* 360**,** 2310-8.

O'CONNELL, Y., MCKENNA, T. J. & CUNNINGHAM, S. K. 1994. The effect of prolactin, human chorionic gonadotropin, insulin and insulin-like growth factor 1 on adrenal steroidogenesis in isolated guinea-pig adrenal cells. *J Steroid Biochem Mol Biol,* 48**,** 235-40.

O'MEARA, N. M., BLACKMAN, J. D., EHRMANN, D. A., BARNES, R. B., JASPAN, J. B., ROSENFIELD, R. L. & POLONSKY, K. S. 1993. Defects in beta-cell function in functional ovarian hyperandrogenism. *J Clin Endocrinol Metab,* 76**,** 1241-7.

O'REILLY, M. W., HOUSE, P. J. & TOMLINSON, J. W. 2014a. Understanding androgen action in adipose tissue. *J Steroid Biochem Mol Biol,* 143**,** 277-84.

O'REILLY, M. W., TAYLOR, A. E., CRABTREE, N. J., HUGHES, B. A., CAPPER, F., CROWLEY, R. K., STEWART, P. M., TOMLINSON, J. W. & ARLT, W. 2014b. Hyperandrogenemia predicts metabolic phenotype in polycystic ovary syndrome: the utility of serum androstenedione. *J Clin Endocrinol Metab***,** jc20133399.

OELKERS, W., DIEDERICH, S. & BAHR, V. 1992. Diagnosis and therapy surveillance in Addison's disease: rapid adrenocorticotropin (ACTH) test and measurement of plasma ACTH, renin activity, and aldosterone. *J Clin Endocrinol Metab,* 75**,** 259-64.

OH, J. Y., BARRETT-CONNOR, E., WEDICK, N. M., WINGARD, D. L. & RANCHO BERNARDO, S. 2002. Endogenous sex hormones and the development of type 2 diabetes in older men and women: the Rancho Bernardo study. *Diabetes Care,* 25**,** 55-60.

OOSTDIJK, W., IDKOWIAK, J., MUELLER, J. W., HOUSE, P. J., TAYLOR, A. E., O'REILLY, M. W., HUGHES, B. A., DE VRIES, M. C., KANT, S. G., SANTEN, G. W., VERKERK, A. J., UITTERLINDEN, A. G., WIT, J. M., LOSEKOOT, M. & ARLT, W. 2015. PAPSS2 deficiency causes androgen excess via impaired DHEA sulfation - in vitro and in vivo studies in a family harboring two novel PAPSS2 mutations. *J Clin Endocrinol Metab***,** jc20143556.

OPPENHEIMER, E., LINDER, B. & DIMARTINO-NARDI, J. 1995. Decreased insulin sensitivity in prepubertal girls with premature adrenarche and acanthosis nigricans. *J Clin Endocrinol Metab,* 80**,** 614-8.

ORENTREICH, N., BRIND, J. L., VOGELMAN, J. H., ANDRES, R. & BALDWIN, H. 1992. Long-term longitudinal measurements of plasma dehydroepiandrosterone sulfate in normal men. *J Clin Endocrinol Metab,* 75**,** 1002-4.

ORIO, F., JR., MATARESE, G., DI BIASE, S., PALOMBA, S., LABELLA, D., SANNA, V., SAVASTANO, S., ZULLO, F., COLAO, A. & LOMBARDI, G. 2003. Exon 6 and 2 peroxisome proliferator-activated receptor-gamma polymorphisms in polycystic ovary syndrome. *J Clin Endocrinol Metab,* 88**,** 5887-92.

OTTOSSON, M., MARIN, P., KARASON, K., ELANDER, A. & BJORNTORP, P. 1995. Blockade of the glucocorticoid receptor with RU 486: effects in vitro and in vivo on human adipose tissue lipoprotein lipase activity. *Obes Res,* 3**,** 233-40.

PACHE, T. D., DE JONG, F. H., HOP, W. C. & FAUSER, B. C. 1993. Association between ovarian changes assessed by transvaginal sonography and clinical and endocrine signs of the polycystic ovary syndrome. *Fertil Steril,* 59**,** 544-9.

PADMANABHAN, V. & VEIGA-LOPEZ, A. 2013. Sheep models of polycystic ovary syndrome phenotype. *Mol Cell Endocrinol,* 373**,** 8-20.

PADMANABHAN, V., VEIGA-LOPEZ, A., ABBOTT, D. H., RECABARREN, S. E. & HERKIMER, C. 2010. Developmental programming: impact of prenatal testosterone excess and postnatal weight gain on insulin sensitivity index and transfer of traits to offspring of overweight females. *Endocrinology,* 151**,** 595-605.

PANIDIS, D., MACUT, D., FARMAKIOTIS, D., ROUSSO, D., KOURTIS, A., KATSIKIS, I., SPANOS, N., PETAKOV, M., BJEKIC, J. & DAMJANOVIC, S. 2006. Indices of insulin sensitivity, beta cell function and serum proinsulin levels in the polycystic ovary syndrome. *Eur J Obstet Gynecol Reprod Biol,* 127**,** 99-105.

PARADISI, R. & VENTUROLI, S. 2010. Retrospective observational study on the effects and tolerability of flutamide in a large population of patients with various kinds of hirsutism over a 15-year period. *Eur J Endocrinol,* 163**,** 139-47.

PARDRIDGE, W. M. 1986. Serum bioavailability of sex steroid hormones. *Clin Endocrinol Metab,* 15**,** 259-78.

PARKER, V. E. & SEMPLE, R. K. 2013. Genetics in endocrinology: genetic forms of severe insulin resistance: what endocrinologists should know. *Eur J Endocrinol,* 169**,** R71-80.

PASQUALI, R., CASIMIRRI, F., CANTOBELLI, S., MELCHIONDA, N., MORSELLI LABATE, A. M., FABBRI, R., CAPELLI, M. & BORTOLUZZI, L. 1991. Effect of obesity and body fat distribution on sex hormones and insulin in men. *Metabolism,* 40**,** 101-4.

PECQUERY, R., LENEVEU, M. C. & GIUDICELLI, Y. 1988. Influence of androgenic status on the alpha 2/beta-adrenergic control of lipolysis in white fat cells: predominant alpha 2-antilipolytic response in testosterone-treated-castrated hamsters. *Endocrinology,* 122**,** 2590-6.

PENNING, T. M., BURCZYNSKI, M. E., JEZ, J. M., HUNG, C. F., LIN, H. K., MA, H., MOORE, M., PALACKAL, N. & RATNAM, K. 2000. Human 3alpha-hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reductase superfamily: functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. *Biochem J,* 351**,** 67-77.

PERRY, R. J., SAMUEL, V. T., PETERSEN, K. F. & SHULMAN, G. I. 2014. The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes. *Nature,* 510**,** 84-91.

PLYMATE, S. R., MATEJ, L. A., JONES, R. E. & FRIEDL, K. E. 1988. Inhibition of sex hormone-binding globulin production in the human hepatoma (Hep G2) cell line by insulin and prolactin. *J Clin Endocrinol Metab,* 67**,** 460-4.

POISSONNET, C. M., BURDI, A. R. & GARN, S. M. 1984. The chronology of adipose tissue appearance and distribution in the human fetus. *Early Hum Dev,* 10**,** 1-11.

POLDERMAN, K. H., GOOREN, L. J., ASSCHEMAN, H., BAKKER, A. & HEINE, R. J. 1994. Induction of insulin resistance by androgens and estrogens. *J Clin Endocrinol Metab,* 79**,** 265-71.

POLSON, D. W., ADAMS, J., WADSWORTH, J. & FRANKS, S. 1988. Polycystic ovaries--a common finding in normal women. *Lancet,* 1**,** 870-2.

POND, C. M. 1992. An evolutionary and functional view of mammalian adipose tissue. *Proc Nutr Soc,* 51**,** 367-77.

PORETSKY, L. 1991. On the paradox of insulin-induced hyperandrogenism in insulin-resistant states. *Endocr Rev,* 12**,** 3-13.

PORETSKY, L., CATALDO, N. A., ROSENWAKS, Z. & GIUDICE, L. C. 1999. The insulin-related ovarian regulatory system in health and disease. *Endocr Rev,* 20**,** 535-82.

PRIEUR, X., LE MAY, C., MAGRE, J. & CARIOU, B. 2014. Congenital lipodystrophies and dyslipidemias. *Curr Atheroscler Rep,* 16**,** 437.

PUCHE, C., JOSE, M., CABERO, A. & MESEGUER, A. 2002. Expression and enzymatic activity of the P450c17 gene in human adipose tissue. *Eur J Endocrinol,* 146**,** 223-9.

PUGEAT, M., DUCLUZEAU, P. H. & MALLION-DONADIEU, M. 2000. Association of insulin resistance with hyperandrogenia in women. *Horm Res,* 54**,** 322-6.

QI, L., HEREDIA, J. E., ALTAREJOS, J. Y., SCREATON, R., GOEBEL, N., NIESSEN, S., MACLEOD, I. X., LIEW, C. W., KULKARNI, R. N., BAIN, J., NEWGARD, C., NELSON, M., EVANS, R. M., YATES, J. & MONTMINY, M. 2006. TRB3 links the E3 ubiquitin ligase COP1 to lipid metabolism. *Science,* 312**,** 1763-6.

QI, Y., XU, Z., ZHU, Q., THOMAS, C., KUMAR, R., FENG, H., DOSTAL, D. E., WHITE, M. F., BAKER, K. M. & GUO, S. 2013. Myocardial loss of IRS1 and IRS2 causes heart failure and is controlled by p38alpha MAPK during insulin resistance. *Diabetes,* 62**,** 3887-900.

QIN, K., EHRMANN, D. A., COX, N., REFETOFF, S. & ROSENFIELD, R. L. 2006. Identification of a functional polymorphism of the human type 5 17beta-hydroxysteroid dehydrogenase gene associated with polycystic ovary syndrome. *The Journal of clinical endocrinology and metabolism,* 91**,** 270-6.

QUINKLER, M., SINHA, B., TOMLINSON, J. W., BUJALSKA, I. J., STEWART, P. M. & ARLT, W. 2004. Androgen generation in adipose tissue in women with simple obesity--a site-specific role for 17beta-hydroxysteroid dehydrogenase type 5. *The Journal of endocrinology,* 183**,** 331-42.

RABSON, S. M. & MENDENHALL, E. N. 1956. Familial hypertrophy of pineal body, hyperplasia of adrenal cortex and diabetes mellitus; report of 3 cases. *Am J Clin Pathol,* 26**,** 283-90.

RAE, M., GRACE, C., HOGG, K., WILSON, L. M., MCHAFFIE, S. L., RAMASWAMY, S., MACCALLUM, J., CONNOLLY, F., MCNEILLY, A. S. & DUNCAN, C. 2013. The pancreas is altered by in utero androgen exposure: implications for clinical conditions such as polycystic ovary syndrome (PCOS). *PLoS One,* 8**,** e56263.

RAMEZANI TEHRANI, F., MINOOEE, S. & AZIZI, F. 2014. Comparison of various adiposity indexes in women with polycystic ovary syndrome and normo-ovulatory non-hirsute women: a population-based study. *Eur J Endocrinol,* 171**,** 199-207.

RAO, P. M., KELLY, D. M. & JONES, T. H. 2013. Testosterone and insulin resistance in the metabolic syndrome and T2DM in men. *Nat Rev Endocrinol,* 9**,** 479-93.

REBUFFE-SCRIVE, M., MARIN, P. & BJORNTORP, P. 1991. Effect of testosterone on abdominal adipose tissue in men. *Int J Obes,* 15**,** 791-5.

REICHERT, M. & EICK, D. 1999. Analysis of cell cycle arrest in adipocyte differentiation. *Oncogene,* 18**,** 459-66.

REMER, T., BOYE, K. R., HARTMANN, M. F. & WUDY, S. A. 2005. Urinary markers of adrenarche: reference values in healthy subjects, aged 3-18 years. *J Clin Endocrinol Metab,* 90**,** 2015-21.

RHODES, C. J. & WHITE, M. F. 2002. Molecular insights into insulin action and secretion. *Eur J Clin Invest,* 32 Suppl 3**,** 3-13.

RISTOW, M., MULLER-WIELAND, D., PFEIFFER, A., KRONE, W. & KAHN, C. R. 1998. Obesity associated with a mutation in a genetic regulator of adipocyte differentiation. *N Engl J Med,* 339**,** 953-9.

RITTMASTER, R. S. & THOMPSON, D. L. 1990. Effect of leuprolide and dexamethasone on hair growth and hormone levels in hirsute women: the relative importance of the

ovary and the adrenal in the pathogenesis of hirsutism. *J Clin Endocrinol Metab,* 70**,** 1096-102.

RODBELL, M. 1964. Metabolism of Isolated Fat Cells. I. Effects of Hormones on Glucose Metabolism and Lipolysis. *J Biol Chem,* 239**,** 375-80.

RODRIGUEZ-CUENCA, S., MONJO, M., PROENZA, A. M. & ROCA, P. 2005. Depot differences in steroid receptor expression in adipose tissue: possible role of the local steroid milieu. *American journal of physiology. Endocrinology and metabolism,* 288**,** E200-7.

ROLAND, A. V., NUNEMAKER, C. S., KELLER, S. R. & MOENTER, S. M. 2010. Prenatal androgen exposure programs metabolic dysfunction in female mice. *J Endocrinol,* 207**,** 213-23.

ROMUALDI, D., GIULIANI, M., DRAISCI, G., COSTANTINI, B., CRISTELLO, F., LANZONE, A. & GUIDO, M. 2007. Pioglitazone reduces the adrenal androgen response to corticotropin-releasing factor without changes in ACTH release in hyperinsulinemic women with polycystic ovary syndrome. *Fertil Steril,* 88**,** 131-8.

ROSATO, E. F., VEMULAPALLI, P., LANG, C. H. & LANZA-JACOBY, S. 1997. Insulin stimulates lipoprotein lipase activity and synthesis in adipocytes from septic rats. *J Surg Res,* 73**,** 73-9.

ROSENFIELD, R. L., MORTENSEN, M., WROBLEWSKI, K., LITTLEJOHN, E. & EHRMANN, D. A. 2011. Determination of the source of androgen excess in functionally atypical polycystic ovary syndrome by a short dexamethasone androgen-suppression test and a low-dose ACTH test. *Hum Reprod,* 26**,** 3138-46.

ROSIGNOLI, C., NICOLAS, J. C., JOMARD, A. & MICHEL, S. 2003. Involvement of the SREBP pathway in the mode of action of androgens in sebaceous glands in vivo. *Exp Dermatol,* 12**,** 480-9.

ROSNER, W., AUCHUS, R. J., AZZIZ, R., SLUSS, P. M. & RAFF, H. 2007. Position statement: Utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement. *The Journal of clinical endocrinology and metabolism,* 92**,** 405-13.

ROTTERDAM, E. A.-S. P. C. W. G. 2004a. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Fertil Steril,* 81**,** 19-25.

ROTTERDAM, E. A.-S. P. C. W. G. 2004b. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod,* 19**,** 41-7.

RUDERMAN, N. B., SAHA, A. K. & KRAEGEN, E. W. 2003. Minireview: malonyl CoA, AMP-activated protein kinase, and adiposity. *Endocrinology,* 144**,** 5166-71.

RUI, L. 2014. Energy metabolism in the liver. *Compr Physiol,* 4**,** 177-97.

RUSSELL, D. W. & WILSON, J. D. 1994. Steroid 5 alpha-reductase: two genes/two enzymes. *Annu Rev Biochem,* 63**,** 25-61.

SARBASSOV, D. D., GUERTIN, D. A., ALI, S. M. & SABATINI, D. M. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science,* 307**,** 1098-101.

SAYGILI, F., OGE, A. & YILMAZ, C. 2005. Hyperinsulinemia and insulin insensitivity in women with nonclassical congenital adrenal hyperplasia due to 21-hydroxylase deficiency: the relationship between serum leptin levels and chronic hyperinsulinemia. *Horm Res,* 63**,** 270-4.

SCHMIDT, J., LANDIN-WILHELMSEN, K., BRANNSTROM, M. & DAHLGREN, E. 2011. Cardiovascular disease and risk factors in PCOS women of postmenopausal age: a 21-year controlled follow-up study. *J Clin Endocrinol Metab,* 96**,** 3794-803.

SCHULTZE, S. M., JENSEN, J., HEMMINGS, B. A., TSCHOPP, O. & NIESSEN, M. 2011. Promiscuous affairs of PKB/AKT isoforms in metabolism. *Arch Physiol Biochem,* 117**,** 70-7.

SEIDELL, J. C., BJORNTORP, P., SJOSTROM, L., KVIST, H. & SANNERSTEDT, R. 1990. Visceral fat accumulation in men is positively associated with insulin, glucose, and C-peptide levels, but negatively with testosterone levels. *Metabolism,* 39**,** 897-901.

SEMPLE, R. K., SAVAGE, D. B., COCHRAN, E. K., GORDEN, P. & O'RAHILLY, S. 2011. Genetic syndromes of severe insulin resistance. *Endocr Rev,* 32**,** 498-514.

SEMPLE, R. K., SLEIGH, A., MURGATROYD, P. R., ADAMS, C. A., BLUCK, L., JACKSON, S., VOTTERO, A., KANABAR, D., CHARLTON-MENYS, V., DURRINGTON, P., SOOS, M. A., CARPENTER, T. A., LOMAS, D. J., COCHRAN, E. K., GORDEN, P., O'RAHILLY, S. & SAVAGE, D. B. 2009. Postreceptor insulin resistance contributes to human dyslipidemia and hepatic steatosis. *J Clin Invest,* 119**,** 315-22.

SEMPLE, R. K., WILLIAMS, R. M. & DUNGER, D. B. 2010. What is the best management strategy for patients with severe insulin resistance? *Clin Endocrinol (Oxf),* 73**,** 286-90.

SERRANO, R., VILLAR, M., MARTINEZ, C., CARRASCOSA, J. M., GALLARDO, N. & ANDRES, A. 2005. Differential gene expression of insulin receptor isoforms A and B and insulin receptor substrates 1, 2 and 3 in rat tissues: modulation by aging and differentiation in rat adipose tissue. *J Mol Endocrinol,* 34**,** 153-61.

SEWTER, C., BERGER, D., CONSIDINE, R. V., MEDINA, G., ROCHFORD, J., CIARALDI, T., HENRY, R., DOHM, L., FLIER, J. S., O'RAHILLY, S. & VIDAL-PUIG, A. J. 2002. Human obesity and type 2 diabetes are associated with alterations in SREBP1 isoform expression that are reproduced ex vivo by tumor necrosis factor-alpha. *Diabetes,* 51**,** 1035-41.

SHACKLETON, C. H. 1993. Mass spectrometry in the diagnosis of steroid-related disorders and in hypertension research. *J Steroid Biochem Mol Biol,* 45**,** 127-40.

SHAW, L. J., BAIREY MERZ, C. N., AZZIZ, R., STANCZYK, F. Z., SOPKO, G., BRAUNSTEIN, G. D., KELSEY, S. F., KIP, K. E., COOPER-DEHOFF, R. M., JOHNSON, B. D., VACCARINO, V., REIS, S. E., BITTNER, V., HODGSON, T. K., ROGERS, W. & PEPINE, C. J. 2008. Postmenopausal women with a history of irregular menses and elevated androgen measurements at high risk for worsening cardiovascular event-free survival: results from the National Institutes of Health--National Heart, Lung, and Blood Institute sponsored Women's Ischemia Syndrome Evaluation. *J Clin Endocrinol Metab,* 93**,** 1276-84.

SHIBUYA, R., SUZUKI, T., MIKI, Y., YOSHIDA, K., MORIYA, T., ONO, K., AKAHIRA, J., ISHIDA, T., HIRAKAWA, H., EVANS, D. B. & SASANO, H. 2008. Intratumoral concentration of sex steroids and expression of sex steroid-producing enzymes in ductal carcinoma in situ of human breast. *Endocr Relat Cancer,* 15**,** 113-24.

SHIFREN, J. L., BRAUNSTEIN, G. D., SIMON, J. A., CASSON, P. R., BUSTER, J. E., REDMOND, G. P., BURKI, R. E., GINSBURG, E. S., ROSEN, R. C., LEIBLUM, S. R., CARAMELLI, K. E. & MAZER, N. A. 2000. Transdermal testosterone treatment in women with impaired sexual function after oophorectomy. *N Engl J Med,* 343**,** 682-8.

SHIMANO, H., YAHAGI, N., AMEMIYA-KUDO, M., HASTY, A. H., OSUGA, J., TAMURA, Y., SHIONOIRI, F., IIZUKA, Y., OHASHI, K., HARADA, K., GOTODA, T., ISHIBASHI, S. & YAMADA, N. 1999. Sterol regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes. *J Biol Chem,* 274**,** 35832-9.

SHULMAN, G. I. 2014. Ectopic fat in insulin resistance, dyslipidemia, and cardiometabolic disease. *N Engl J Med,* 371**,** 2237-8.

SILFEN, M. E., SHACKLETON, C. H., MANIBO, A. M., LEVINE, L. S., SEKHAR, D., MCMAHON, D. J. & OBERFIELD, S. E. 2002. 5 alpha-reductase and 11 beta-hydroxysteroid dehydrogenase activity in prepubertal Hispanic girls with premature adrenarche. *J Clin Endocrinol Metab,* 87**,** 4647-51.

SINGH, R., ARTAZA, J. N., TAYLOR, W. E., BRAGA, M., YUAN, X., GONZALEZ-CADAVID, N. F. & BHASIN, S. 2006. Testosterone inhibits adipogenic differentiation in 3T3-L1 cells: nuclear translocation of androgen receptor complex with beta-catenin and T-cell factor 4 may bypass canonical Wnt signaling to down-regulate adipogenic transcription factors. *Endocrinology,* 147**,** 141-54.

SINGH, R., ARTAZA, J. N., TAYLOR, W. E., GONZALEZ-CADAVID, N. F. & BHASIN, S. 2003. Androgens stimulate myogenic differentiation and inhibit adipogenesis in C3H 10T1/2 pluripotent cells through an androgen receptor-mediated pathway. *Endocrinology,* 144**,** 5081-8.

SIR-PETERMANN, T., HITCHSFELD, C., MALIQUEO, M., CODNER, E., ECHIBURU, B., GAZITUA, R., RECABARREN, S. & CASSORLA, F. 2005. Birth weight in offspring of mothers with polycystic ovarian syndrome. *Hum Reprod,* 20**,** 2122-6.

SKLAR, C. A., KAPLAN, S. L. & GRUMBACH, M. M. 1980. Evidence for dissociation between adrenarche and gonadarche: studies in patients with idiopathic precocious puberty, gonadal dysgenesis, isolated gonadotropin deficiency, and constitutionally delayed growth and adolescence. *J Clin Endocrinol Metab,* 51**,** 548-56.

SMAS, C. M., CHEN, L., ZHAO, L., LATASA, M. J. & SUL, H. S. 1999. Transcriptional repression of pref-1 by glucocorticoids promotes 3T3-L1 adipocyte differentiation. *J Biol Chem,* 274**,** 12632-41.

SMAS, C. M. & SUL, H. S. 1997. Molecular mechanisms of adipocyte differentiation and inhibitory action of pref-1. *Crit Rev Eukaryot Gene Expr,* 7**,** 281-98.

SMITH, P. J., WISE, L. S., BERKOWITZ, R., WAN, C. & RUBIN, C. S. 1988. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. *J Biol Chem,* 263**,** 9402-8.

SMITH, S., RAVNIKAR, V. A. & BARBIERI, R. L. 1987. Androgen and insulin response to an oral glucose challenge in hyperandrogenic women. *Fertil Steril,* 48**,** 72-7.

SMITH, S. R., LOVEJOY, J. C., GREENWAY, F., RYAN, D., DEJONGE, L., DE LA BRETONNE, J., VOLAFOVA, J. & BRAY, G. A. 2001. Contributions of total body fat, abdominal subcutaneous adipose tissue compartments, and visceral adipose tissue to the metabolic complications of obesity. *Metabolism,* 50**,** 425-35.

SOETERS, M. R., SAUERWEIN, H. P., GROENER, J. E., AERTS, J. M., ACKERMANS, M. T., GLATZ, J. F., FLIERS, E. & SERLIE, M. J. 2007. Gender-related differences in the metabolic response to fasting. *The Journal of clinical endocrinology and metabolism,* 92**,** 3646-52.

SOULE, S. G., CONWAY, G., PRELEVIC, G. M., PRENTICE, M., GINSBURG, J. & JACOBS, H. S. 1995. Osteopenia as a feature of the androgen insensitivity syndrome. *Clin Endocrinol (Oxf),* 43**,** 671-5.

SPEISER, P. W., SERRAT, J., NEW, M. I. & GERTNER, J. M. 1992. Insulin insensitivity in adrenal hyperplasia due to nonclassical steroid 21-hydroxylase deficiency. *J Clin Endocrinol Metab,* 75**,** 1421-4.

SPEISER, P. W. & WHITE, P. C. 2003. Congenital adrenal hyperplasia. *N Engl J Med,* 349**,** 776-88.

SPIEGELMAN, B. M. & FARMER, S. R. 1982. Decreases in tubulin and actin gene expression prior to morphological differentiation of 3T3 adipocytes. *Cell,* 29**,** 53-60.

SRINATH, R., HILL GOLDEN, S., CARSON, K. A. & DOBS, A. 2015. Endogenous Testosterone and its relationship to preclinical and clinical measures of cardiovascular disease in the Atherosclerosis Risk in Communities (ARIC) Study. *J Clin Endocrinol Metab***,** jc20143934.

STEINER, D. F., CHAN, S. J., WELSH, J. M. & KWOK, S. C. 1985. Structure and evolution of the insulin gene. *Annu Rev Genet,* 19**,** 463-84.

STEWART, P. M., BOULTON, A., KUMAR, S., CLARK, P. M. & SHACKLETON, C. H. 1999. Cortisol metabolism in human obesity: impaired cortisone-->cortisol conversion in subjects with central adiposity. *J Clin Endocrinol Metab,* 84**,** 1022-7.

STEWART, P. M., SHACKLETON, C. H., BEASTALL, G. H. & EDWARDS, C. R. 1990. 5 alpha-reductase activity in polycystic ovary syndrome. *Lancet,* 335**,** 431-3.

STIMSON, R. H., JOHNSTONE, A. M., HOMER, N. Z., WAKE, D. J., MORTON, N. M., ANDREW, R., LOBLEY, G. E. & WALKER, B. R. 2007. Dietary macronutrient content alters cortisol metabolism independently of body weight changes in obese men. *J Clin Endocrinol Metab,* 92**,** 4480-4.

STRAWFORD, A., ANTELO, F., CHRISTIANSEN, M. & HELLERSTEIN, M. K. 2004. Adipose tissue triglyceride turnover, de novo lipogenesis, and cell proliferation in humans measured with 2H2O. *Am J Physiol Endocrinol Metab,* 286**,** E577-88.

STUBBS, S. A., WEBBER, L. J., STARK, J., RICE, S., MARGARA, R., LAVERY, S., TREW, G. H., HARDY, K. & FRANKS, S. 2013. Role of Insulin-like growth factors in initiation of follicle growth in normal and polycystic human ovaries. *J Clin Endocrinol Metab,* 98**,** 3298-305.

STUDEN, K. B., SEBESTJEN, M., PFEIFER, M. & PREZELJ, J. 2011. Influence of spironolactone treatment on endothelial function in non-obese women with polycystic ovary syndrome. *European journal of endocrinology / European Federation of Endocrine Societies,* 164**,** 389-95.

STUMVOLL, M., MITRAKOU, A., PIMENTA, W., JENSSEN, T., YKI-JARVINEN, H., VAN HAEFTEN, T., RENN, W. & GERICH, J. 2000. Use of the oral glucose tolerance test to assess insulin release and insulin sensitivity. *Diabetes care,* 23**,** 295-301.

SUN, L., STENKEN, J. A., BRUNNER, J. E., MICHEL, K. B., ADELSBERGER, J. K., YANG, A. Y., ZHAO, J. J. & MUSSON, D. G. 2008. An in vivo microdialysis coupled with liquid chromatography/tandem mass spectrometry study of cortisol metabolism in monkey adipose tissue. *Anal Biochem,* 381**,** 214-23.

SVERRISDOTTIR, Y. B., MOGREN, T., KATAOKA, J., JANSON, P. O. & STENER-VICTORIN, E. 2008. Is polycystic ovary syndrome associated with high sympathetic nerve activity and size at birth? *Am J Physiol Endocrinol Metab,* 294**,** E576-81.

SWIGLO, B. A., COSMA, M., FLYNN, D. N., KURTZ, D. M., LABELLA, M. L., MULLAN, R. J., ERWIN, P. J. & MONTORI, V. M. 2008. Clinical review: Antiandrogens for the treatment of hirsutism: a systematic review and metaanalyses of randomized controlled trials. *J Clin Endocrinol Metab,* 93**,** 1153-60.

TAPONEN, S., MARTIKAINEN, H., JARVELIN, M. R., SOVIO, U., LAITINEN, J., POUTA, A., HARTIKAINEN, A. L., MCCARTHY, M. I., FRANKS, S., PALDANIUS, M. & RUOKONEN, A. 2004. Metabolic cardiovascular disease risk factors in women with self-reported symptoms of oligomenorrhea and/or hirsutism: Northern Finland Birth Cohort 1966 Study. *The Journal of clinical endocrinology and metabolism,* 89**,** 2114-8.

TEHRANI, F. R., NOROOZZADEH, M., ZAHEDIASL, S., PIRYAEI, A. & AZIZI, F. 2014. Introducing a rat model of prenatal androgen-induced polycystic ovary syndrome in adulthood. *Exp Physiol,* 99**,** 792-801.

TEICHERT-KULISZEWSKA, K., HAMILTON, B. S., DEITEL, M. & RONCARI, D. A. 1992. Augmented production of heparin-binding mitogenic proteins by preadipocytes from massively obese persons. *J Clin Invest,* 90**,** 1226-31.

TEIXEIRA, R. J., GINZBARG, D., RODRIGUES FREITAS, J., FUCKS, G., SILVA, C. M. & BORDALLO, M. A. 2004. Serum leptin levels in premature pubarche and prepubertal girls with and without obesity. *J Pediatr Endocrinol Metab,* 17**,** 1393-8.

THESSALONIKI, E. A.-S. P. C. W. G. 2008. Consensus on infertility treatment related to polycystic ovary syndrome. *Fertil Steril,* 89**,** 505-22.

THOMPSON, A. L., LIM-FRASER, M. Y., KRAEGEN, E. W. & COONEY, G. J. 2000. Effects of individual fatty acids on glucose uptake and glycogen synthesis in soleus muscle in vitro. *Am J Physiol Endocrinol Metab,* 279**,** E577-84.

TIITINEN, A., PEKONEN, F., STENMAN, U. H. & LAATIKAINEN, T. 1990. Plasma androgens and oestradiol during oral glucose tolerance test in patients with polycystic ovaries. *Hum Reprod,* 5**,** 242-5.

TOMLINSON, J. W., FINNEY, J., GAY, C., HUGHES, B. A., HUGHES, S. V. & STEWART, P. M. 2008a. Impaired glucose tolerance and insulin resistance are associated with increased adipose 11beta-hydroxysteroid dehydrogenase type 1 expression and elevated hepatic 5alpha-reductase activity. *Diabetes,* 57**,** 2652-60.

TOMLINSON, J. W., FINNEY, J., HUGHES, B. A., HUGHES, S. V. & STEWART, P. M. 2008b. Reduced glucocorticoid production rate, decreased 5alpha-reductase activity, and adipose tissue insulin sensitization after weight loss. *Diabetes,* 57**,** 1536-43.

TOMLINSON, J. W., SHERLOCK, M., HUGHES, B., HUGHES, S. V., KILVINGTON, F., BARTLETT, W., COURTNEY, R., REJTO, P., CARLEY, W. & STEWART, P. M. 2007. Inhibition of 11beta-hydroxysteroid dehydrogenase type 1 activity in vivo limits glucocorticoid exposure to human adipose tissue and decreases lipolysis. *J Clin Endocrinol Metab,* 92**,** 857-64.

TOSI, F., NEGRI, C., PERRONE, F., DORIZZI, R., CASTELLO, R., BONORA, E. & MOGHETTI, P. 2012. Hyperinsulinemia amplifies GnRH agonist stimulated ovarian steroid secretion in women with polycystic ovary syndrome. *The Journal of clinical endocrinology and metabolism,* 97**,** 1712-9.

TRIGATTI, B. L., ANDERSON, R. G. & GERBER, G. E. 1999. Identification of caveolin-1 as a fatty acid binding protein. *Biochem Biophys Res Commun,* 255**,** 34-9.

TSILCHOROZIDOU, T., HONOUR, J. W. & CONWAY, G. S. 2003. Altered cortisol metabolism in polycystic ovary syndrome: insulin enhances 5alpha-reduction but not the elevated adrenal steroid production rates. *J Clin Endocrinol Metab,* 88**,** 5907-13.

TURNBOW, M. A., KELLER, S. R., RICE, K. M. & GARNER, C. W. 1994. Dexamethasone down-regulation of insulin receptor substrate-1 in 3T3-L1 adipocytes. *J Biol Chem,* 269**,** 2516-20.

UTRIAINEN, P., JAASKELAINEN, J., ROMPPANEN, J. & VOUTILAINEN, R. 2007. Childhood metabolic syndrome and its components in premature adrenarche. *J Clin Endocrinol Metab,* 92**,** 4282-5.

VALLE, L. D., TOFFOLO, V., NARDI, A., FIORE, C., BERNANTE, P., DI LIDDO, R., PARNIGOTTO, P. P. & COLOMBO, L. 2006. Tissue-specific transcriptional initiation and activity of steroid sulfatase complementing dehydroepiandrosterone sulfate uptake and intracrine steroid activations in human adipose tissue. *The Journal of endocrinology,* 190**,** 129-39.

VAN HOUTEN, E. L., KRAMER, P., MCLUSKEY, A., KARELS, B., THEMMEN, A. P. & VISSER, J. A. 2012. Reproductive and metabolic phenotype of a mouse model of PCOS. *Endocrinology,* 153**,** 2861-9.

VARLAMOV, O., BETHEA, C. L. & ROBERTS, C. T., JR. 2014. Sex-specific differences in lipid and glucose metabolism. *Front Endocrinol (Lausanne),* 5**,** 241.

VARLAMOV, O., CHU, M. P., MCGEE, W. K., CAMERON, J. L., O'ROURKE, R. W., MEYER, K. A., BISHOP, C. V., STOUFFER, R. L. & ROBERTS, C. T., JR. 2013. Ovarian cycle-specific regulation of adipose tissue lipid storage by testosterone in female nonhuman primates. *Endocrinology,* 154**,** 4126-35.

VARLAMOV, O., WHITE, A. E., CARROLL, J. M., BETHEA, C. L., REDDY, A., SLAYDEN, O., O'ROURKE, R. W. & ROBERTS, C. T., JR. 2012. Androgen effects on adipose tissue architecture and function in nonhuman primates. *Endocrinology,* 153**,** 3100-10.

VASSILIADI, D. A., BARBER, T. M., HUGHES, B. A., MCCARTHY, M. I., WASS, J. A., FRANKS, S., NIGHTINGALE, P., TOMLINSON, J. W., ARLT, W. & STEWART, P. M. 2009. Increased 5 alpha-reductase activity and adrenocortical drive in women with polycystic ovary syndrome. *The Journal of clinical endocrinology and metabolism,* 94**,** 3558-66.

VAZQUEZ-VELA, M. E., TORRES, N. & TOVAR, A. R. 2008. White adipose tissue as endocrine organ and its role in obesity. *Arch Med Res,* 39**,** 715-28.

VENDOLA, K., ZHOU, J., WANG, J. & BONDY, C. A. 1999. Androgens promote insulin-like growth factor-I and insulin-like growth factor-I receptor gene expression in the primate ovary. *Hum Reprod,* 14**,** 2328-32.

VERMEULEN, A., VERDONCK, L. & KAUFMAN, J. M. 1999. A critical evaluation of simple methods for the estimation of free testosterone in serum. *J Clin Endocrinol Metab,* 84**,** 3666-72.

VIDAL-PUIG, A., JIMENEZ-LINAN, M., LOWELL, B. B., HAMANN, A., HU, E., SPIEGELMAN, B., FLIER, J. S. & MOLLER, D. E. 1996. Regulation of PPAR gamma gene expression by nutrition and obesity in rodents. *J Clin Invest,* 97**,** 2553-61.

VIDAL-PUIG, A. J., CONSIDINE, R. V., JIMENEZ-LINAN, M., WERMAN, A., PORIES, W. J., CARO, J. F. & FLIER, J. S. 1997. Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. *J Clin Invest,* 99**,** 2416-22.

VILLA, J. & PRATLEY, R. E. 2011. Adipose tissue dysfunction in polycystic ovary syndrome. *Curr Diab Rep,* 11**,** 179-84.

VILLAR, J., CELAY, J., ALONSO, M. M., ROTINEN, M., DE MIGUEL, C., MIGLIACCIO, M. & ENCIO, I. 2007. Transcriptional regulation of the human type 8 17beta-hydroxysteroid dehydrogenase gene by C/EBPbeta. *J Steroid Biochem Mol Biol,* 105**,** 131-9.

VILLENA, J. A., ROY, S., SARKADI-NAGY, E., KIM, K. H. & SUL, H. S. 2004. Desnutrin, an adipocyte gene encoding a novel patatin domain-containing protein, is induced by fasting and glucocorticoids: ectopic expression of desnutrin increases triglyceride hydrolysis. *J Biol Chem,* 279**,** 47066-75.

VITKU, J., CHLUPACOVA, T., SOSVOROVA, L., HAMPL, R., HILL, M., HERACEK, J., BICIKOVA, M. & STARKA, L. 2015. Development and validation of LC-MS/MS method for quantification of bisphenol A and estrogens in human plasma and seminal fluid. *Talanta,* 140**,** 62-7.

VOUTILAINEN, R., PERHEENTUPA, J. & APTER, D. 1983. Benign premature adrenarche: clinical features and serum steroid levels. *Acta Paediatr Scand,* 72**,** 707-11.

WABITSCH, M., BRENNER, R. E., MELZNER, I., BRAUN, M., MOLLER, P., HEINZE, E., DEBATIN, K. M. & HAUNER, H. 2001. Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation. *Int J Obes Relat Metab Disord,* 25**,** 8-15.

WAKE, D. J., STRAND, M., RASK, E., WESTERBACKA, J., LIVINGSTONE, D. E., SODERBERG, S., ANDREW, R., YKI-JARVINEN, H., OLSSON, T. & WALKER, B. R. 2007. Intra-adipose sex steroid metabolism and body fat distribution in idiopathic human obesity. *Clin Endocrinol (Oxf),* 66**,** 440-6.

WALTERS, K. A., ALLAN, C. M. & HANDELSMAN, D. J. 2012. Rodent models for human polycystic ovary syndrome. *Biol Reprod,* 86**,** 149, 1-12.

WANG, F., KOSKELA, A., HAMALAINEN, E., TURPEINEN, U., SAVOLAINEN-PELTONEN, H., MIKKOLA, T. S., VIHMA, V., ADLERCREUTZ, H. & TIKKANEN, M. J. 2011. Quantitative determination of dehydroepiandrosterone fatty acyl esters in human female adipose tissue and serum using mass spectrometric methods. *J Steroid Biochem Mol Biol,* 124**,** 93-8.

WANG, F., VIHMA, V., SORONEN, J., TURPEINEN, U., HAMALAINEN, E., SAVOLAINEN-PELTONEN, H., MIKKOLA, T. S., NAUKKARINEN, J., PIETILAINEN, K. H., JAUHIAINEN, M., YKI-JARVINEN, H. & TIKKANEN, M. J. 2013. 17beta-Estradiol and estradiol fatty acyl esters and estrogen-converting enzyme expression in adipose tissue in obese men and women. *J Clin Endocrinol Metab,* 98**,** 4923-31.

WANG, L., LI, S., ZHAO, A., TAO, T., MAO, X., ZHANG, P. & LIU, W. 2012. The expression of sex steroid synthesis and inactivation enzymes in subcutaneous adipose tissue of PCOS patients. *J Steroid Biochem Mol Biol,* 132**,** 120-6.

WASNIEWSKA, M., BALSAMO, A., VALENZISE, M., MANGANARO, A., FAGGIOLI, G., BOMBACI, S., CONTI, V., FERRI, M., AVERSA, T., CICOGNANI, A. & DE LUCA, F. 2013. Increased large artery intima media thickness in adolescents with either classical or non-classical congenital adrenal hyperplasia. *J Endocrinol Invest,* 36**,** 12-5.

WEBBER, L. J., STUBBS, S., STARK, J., TREW, G. H., MARGARA, R., HARDY, K. & FRANKS, S. 2003. Formation and early development of follicles in the polycystic ovary. *Lancet,* 362**,** 1017-21.

WEIL, S. J., VENDOLA, K., ZHOU, J., ADESANYA, O. O., WANG, J., OKAFOR, J. & BONDY, C. A. 1998. Androgen receptor gene expression in the primate ovary: cellular localization, regulation, and functional correlations. *J Clin Endocrinol Metab,* 83**,** 2479-85.

WELLS, J. C. 2007. Sexual dimorphism of body composition. *Best Pract Res Clin Endocrinol Metab,* 21**,** 415-30.

WELT, C. K., GUDMUNDSSON, J. A., ARASON, G., ADAMS, J., PALSDOTTIR, H., GUDLAUGSDOTTIR, G., INGADOTTIR, G. & CROWLEY, W. F. 2006. Characterizing discrete subsets of polycystic ovary syndrome as defined by the Rotterdam criteria: the impact of weight on phenotype and metabolic features. *J Clin Endocrinol Metab,* 91**,** 4842-8.

WERNER, R., GROTSCH, H. & HIORT, O. 2010. 46,XY disorders of sex development--the undermasculinised male with disorders of androgen action. *Best Pract Res Clin Endocrinol Metab,* 24**,** 263-77.

WIERMAN, M. E., ARLT, W., BASSON, R., DAVIS, S. R., MILLER, K. K., MURAD, M. H., ROSNER, W. & SANTORO, N. 2014. Androgen therapy in women: a reappraisal: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab,* 99**,** 3489-510.

WIJEYARATNE, C. N., BALEN, A. H., BARTH, J. H. & BELCHETZ, P. E. 2002. Clinical manifestations and insulin resistance (IR) in polycystic ovary syndrome (PCOS) among South Asians and Caucasians: is there a difference? *Clin Endocrinol (Oxf),* 57**,** 343-50.

WILLIAMS, R. M., DEEB, A., ONG, K. K., BICH, W., MURGATROYD, P. R., HUGHES, I. A. & ACERINI, C. L. 2010. Insulin sensitivity and body composition in children with classical and nonclassical congenital adrenal hyperplasia. *Clin Endocrinol (Oxf),* 72**,** 155-60.

WILLIS, D. & FRANKS, S. 1995. Insulin action in human granulosa cells from normal and polycystic ovaries is mediated by the insulin receptor and not the type-I insulin-like growth factor receptor. *J Clin Endocrinol Metab,* 80**,** 3788-90.

WILLIS, D., MASON, H., GILLING-SMITH, C. & FRANKS, S. 1996. Modulation by insulin of follicle-stimulating hormone and luteinizing hormone actions in human granulosa cells of normal and polycystic ovaries. *J Clin Endocrinol Metab,* 81**,** 302-9.

WILLIS, D. S., MASON, H. D., WATSON, H. & FRANKS, S. 1998. Developmentally regulated responses of human granulosa cells to insulin-like growth factors (IGFs): IGF-I and IGF-II action mediated via the type-I IGF receptor. *J Clin Endocrinol Metab,* 83**,** 1256-9.

WITHERS, D. J., GUTIERREZ, J. S., TOWERY, H., BURKS, D. J., REN, J. M., PREVIS, S., ZHANG, Y., BERNAL, D., PONS, S., SHULMAN, G. I., BONNER-WEIR, S. & WHITE, M. F. 1998. Disruption of IRS-2 causes type 2 diabetes in mice. *Nature,* 391**,** 900-4.

WORBOYS, S., KOTSOPOULOS, D., TEEDE, H., MCGRATH, B. & DAVIS, S. R. 2001. Evidence that parenteral testosterone therapy may improve endothelium-dependent and -independent vasodilation in postmenopausal women already receiving estrogen. *J Clin Endocrinol Metab,* 86**,** 158-61.

WU, S., DIVALL, S., NWAOPARA, A., RADOVICK, S., WONDISFORD, F., KO, C. & WOLFE, A. 2014. Obesity-induced infertility and hyperandrogenism are corrected by deletion of the insulin receptor in the ovarian theca cell. *Diabetes,* 63**,** 1270-82.

XU, A., CHAN, K. W., HOO, R. L., WANG, Y., TAN, K. C., ZHANG, J., CHEN, B., LAM, M. C., TSE, C., COOPER, G. J. & LAM, K. S. 2005. Testosterone selectively reduces the high molecular weight form of adiponectin by inhibiting its secretion from adipocytes. *J Biol Chem,* 280**,** 18073-80.

XU, X., DE PERGOLA, G. & BJORNTORP, P. 1990. The effects of androgens on the regulation of lipolysis in adipose precursor cells. *Endocrinology,* 126**,** 1229-34.

XU, X. F., DE PERGOLA, G. & BJORNTORP, P. 1991. Testosterone increases lipolysis and the number of beta-adrenoceptors in male rat adipocytes. *Endocrinology,* 128**,** 379-82.

YAMAMOTO, S., KAMINSKY, J. & BOUR, P. 2012. Structure and vibrational motion of insulin from Raman optical activity spectra. *Anal Chem,* 84**,** 2440-51.

YAN, X., DAI, X., WANG, J., ZHAO, N., CUI, Y. & LIU, J. 2013. Prenatal androgen excess programs metabolic derangements in pubertal female rats. *J Endocrinol,* 217**,** 119-29.

YANASE, T., FAN, W., KYOYA, K., MIN, L., TAKAYANAGI, R., KATO, S. & NAWATA, H. 2008. Androgens and metabolic syndrome: lessons from androgen receptor knock out (ARKO) mice. *The Journal of steroid biochemistry and molecular biology,* 109**,** 254-7.

YANG, J. L., ZHANG, C. P., LI, L., HUANG, L., JI, S. Y., LU, C. L., FAN, C. H., CAI, H., REN, Y., HU, Z. Y., GAO, F. & LIU, Y. X. 2010. Testosterone induces redistribution of forkhead box-3a and down-regulation of growth and differentiation factor 9 messenger ribonucleic acid expression at early stage of mouse folliculogenesis. *Endocrinology,* 151**,** 774-82.

YANG, W. S., LEE, W. J., FUNAHASHI, T., TANAKA, S., MATSUZAWA, Y., CHAO, C. L., CHEN, C. L., TAI, T. Y. & CHUANG, L. M. 2001. Weight reduction increases plasma levels of an adipose-derived anti-inflammatory protein, adiponectin. *The Journal of clinical endocrinology and metabolism,* 86**,** 3815-9.

YARROW, J. F., BEGGS, L. A., CONOVER, C. F., MCCOY, S. C., BECK, D. T. & BORST, S. E. 2012. Influence of androgens on circulating adiponectin in male and female rodents. *PloS one,* 7**,** e47315.

YILDIZ, B. O., BOLOUR, S., WOODS, K., MOORE, A. & AZZIZ, R. 2010. Visually scoring hirsutism. *Hum Reprod Update,* 16**,** 51-64.

YILDIZ, B. O., BOZDAG, G., YAPICI, Z., ESINLER, I. & YARALI, H. 2012. Prevalence, phenotype and cardiometabolic risk of polycystic ovary syndrome under different diagnostic criteria. *Hum Reprod,* 27**,** 3067-73.

YING, H., ARAKI, O., FURUYA, F., KATO, Y. & CHENG, S. Y. 2007. Impaired adipogenesis caused by a mutated thyroid hormone alpha1 receptor. *Mol Cell Biol,* 27**,** 2359-71.

YKI-JARVINEN, H. 1984. Sex and insulin sensitivity. *Metabolism,* 33**,** 1011-5.

ZAMMIT, V. A. 1999. The malonyl-CoA-long-chain acyl-CoA axis in the maintenance of mammalian cell function. *Biochem J,* 343 Pt 3**,** 505-15.

ZECHNER, R., ZIMMERMANN, R., EICHMANN, T. O., KOHLWEIN, S. D., HAEMMERLE, G., LASS, A. & MADEO, F. 2012. FAT SIGNALS--lipases and lipolysis in lipid metabolism and signaling. *Cell Metab,* 15**,** 279-91.

ZHANG, H. J., YANG, J., ZHANG, M. N., LIU, C. Q., XU, M., LI, X. J., YANG, S. Y. & LI, X. Y. 2010. Metabolic disorders in newly diagnosed young adult female patients with simple virilizing 21-hydroxylase deficiency. *Endocrine,* 38**,** 260-5.

ZHANG, Y., DUFORT, I., RHEAULT, P. & LUU-THE, V. 2000. Characterization of a human 20alpha-hydroxysteroid dehydrogenase. *Journal of molecular endocrinology,* 25**,** 221-8.

ZHANG, Y., YIN, L. & HILLGARTNER, F. B. 2003. SREBP-1 integrates the actions of thyroid hormone, insulin, cAMP, and medium-chain fatty acids on ACCalpha transcription in hepatocytes. *J Lipid Res,* 44**,** 356-68.

ZHOU, B. R., HUANG, Q. H., XU, Y., WU, D., YIN, Z. Q. & LUO, D. 2012. Dihydrotestosterone induces SREBP-1 expression and lipogenesis through the phosphoinositide 3-kinase/Akt pathway in HaCaT cells. *Lipids Health Dis,* 11**,** 156.

ZOU, C. Y., GONG, Y. & LIANG, J. 2014. Metabolic signaling of insulin secretion by pancreatic beta-cell and its derangement in type 2 diabetes. *Eur Rev Med Pharmacol Sci,* 18**,** 2215-27.

9 Publications arising from this thesis

1. Hyperandrogenaemia predicts metabolic phenotype in polycystic ovary syndrome: the utility of serum androstenedione. **O'Reilly MW**, Taylor AE, Crabtree NJ, Hughes BA, Capper F, Crowley RK, Stewart PM, Tomlinson JW, Arlt W. *J Clin Endocrinol Metab* 2014 Mar; 99(3): 1027036
2. Understanding androgen action in adipose tissue. **O'Reilly MW**, House PJ, Tomlinson JW. *J Steroid Biochem Mol Biol* 2014 Sep; 143: 277-84
3. PAPSS2 deficiency causes androgen excess via impaired DHEA sulfation – in vitro and in vivo studies in a family harboring two novel PAPSS2 mutations. Oostdijk W, Idkowiak J, Mueller JW, House PJ, Taylor AE, **O'Reilly MW**, Hughes BA, de Vries MC, Kant SG, Santen GW, Verkerk AJ, Uitterlinden AG, Wit JM, Losekoot M, Arlt W. *J Clin Endocrinol Metab* 2015 Apr; 100(4): E672-80