Metabolic effects of aromatase inhibition

Fraser Wilson Gibb

Doctor of Philosophy
University of Edinburgh
2014

Abstract

Aromatase, a member of the cytochrome P450 superfamily, catalyses the conversion of androgens to estrogens; specifically, testosterone to estradiol and androstenedione to estrone. Aromatase is widely expressed across a range of tissues and deleterious metabolic effects are observed in both murine aromatase knock-out models and in rare human cases of aromatase deficiency. The effects of pharmacological inhibition of aromatase, as employed in the treatment of breast cancer, are not well characterised. This thesis addresses the hypothesis that aromatase inhibition, and consequent changes in sex steroid hormone action (higher androgen:estrogen ratio), results in disadvantageous changes in body composition and reduced insulin sensitivity.

In a cohort study of 197 community-dwelling men, lower testosterone and SHBG concentrations were observed in those fulfilling criteria for metabolic syndrome, although no relationship with estrogens was observed. The strongest determinant of circulating estrogens was substrate androgen concentration.

A case-control study of aromatase inhibitor treated breast cancer patients and age-matched controls (n=40) demonstrated decreased insulin sensitivity and increased body fat in those treated with aromatase inhibitors; serum leptin concentration and leptin mRNA transcript levels (in subcutaneous adipose tissue) were elevated in this group.

In healthy male volunteers (n=17), 6 weeks of aromatase inhibition (1 mg anastrozole daily) resulted in reduced glucose disposal during a hyperinsulinaemic euglycaemic clamp study, with d2-glucose and d5-glycerol tracers. No effects upon hepatic insulin sensitivity, lipolysis or body composition were noted, although serum leptin concentration was reduced following aromatase inhibitor administration.

In conclusion, aromatase inhibition is associated with increased insulin resistance and, in women, increased body fat. This may be relevant for patients receiving aromatase inhibitor therapy, where more careful monitoring of glucose tolerance may be warranted.

Declaration

I declare that this thesis was written by me and that the data presented represent my own work, with the exceptions listed below:

- Assistance was provided by Kerry McInnes and Karen French in performing PCR experiments.
- Multiplex immunoassays were performed by Rita Upreti.
- Assistance was provided by Sanjay Kothiya and Abdullah Faqehi in performing GC-MS and LC-MS, respectively.

I declare that this work has not been submitted for any other degree.

Fraser Wilson Gibb, Edinburgh 2015

Acknowledgements

I would like to acknowledge the support and incredible patience of my supervisors, Brian Walker and Ruth Andrew.

Within the Endocrinology Unit, several members of staff provided invaluable advice and assistance, including: Kerry McInnes, Karen French, Sanjay Kothiya, Rita Upreti, Natalie Homer, Scott Denham and Abdullah Faqehi.

I would like to thank the staff at the Wellcome Trust Clinical Research Facility (Western General Hospital), particularly Angie Balfour, Marion MacRury, Karen Paterson and Sam Simpson.

I am grateful to the Wellcome Trust for funding this work.

Finally, thanks to Sarah and my family.

Contents

List of Figures

Chapter 1

Chapter 2

Chapter 4

Chapter 5

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

anastrozole and placebo treatment phases

Abbreviations

11βHSD1	11-Beta hydroxysteroid dehydrogenase type 1
17βHSD3	17-Beta hydroxysteroid dehydrogenase type 3
17βHSD5	17-Beta hydroxysteroid dehydrogenase type 5
3α-HSD3	3-Alpha hydroxysteroid dehydrogenase type 3
5α-R	5alpha-reductase
AKR1C2	Aldo-ketoreductase type 1C2
AKR1C3	Aldo-ketoreductase type 1C3
ACC-1	Acetyl-CoA carboxylase-1
ACTH	Adrenocorticotrophic hormone
ADP	Air displacement plethysmography
AMPK	AMP-activated protein kinase
ANcSR1	Ancestral steroid receptor 1
AR	Androgen receptor
ArKO	Aromatase knockout
BIA	Bioelectric impedance analysis
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CT	Computed tomography
DEPC	Diethylpyrocarbonate
DEXA	Dual-energy x-ray absorptiometry
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid

ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERE	Estrogen response element
ERαKO	Estrogen receptor alpha knockout
EST	Estrogen sulfotransferase
FAI	Free androgen index
FAS	Fatty acid synthase
FFM	Fat free mass
FM	Fat mass
GCMS	Gas chromatography – mass spectrometry
GIP	Gastric inhibitory polypeptide
GIR	Glucose infusion rate
GLUT4	Glucose transporter 4
GnRH	Gonadotrophin releasing hormone
HbA1c	Haemoglobin A1c
HDL	High density lipopprotein
HOMA-IR	Homeostatic model assessment – insulin resistance
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy
HSL	Hormone sensitive lipase
IL	Interleukin
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LDL	Low density lipoprotein
LH	Luteinizing hormone

LOH	Late-onset hypogonadism
LPL	Lipoprotein lipase
LXRα	Liver X receptor alpha
M	Glucose disposal rate
MCP	Monocyte-chemoattractant protein
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
m/z	Mass: charge ratio
NEFA	Non-esterified fatty acids
NNT	Number needed to treat
NPY	Neuropeptide Y
OFN	Oxygen free nitrogen
OGTT	Oral glucose tolerance test
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PDE-3B	Phosphodiesterase-3B
PGE2	Prostaglandin E2
POMC	Pro-opiomelanocortin
PPARγ	Peroxisome proliferator-activated receptor gamma
QUICKI	Quantitative insulin sensitivity check index
Ra	Rate of appearance
RBP4	Retinol binding protein 4
Rd	Rate of disappearance
RT	Room temperature

SAT	Subcutaneous adipose tissue
SERCA-2	Sarco/endoplasmic reticulum Ca^{2+} ATPase
SHBG	Sex hormone-binding globulin
SIMD	Scottish index of multiple deprivation
SREBP	Sterol regulatory element-binding protein
T2DM	Type 2 Diabetes Mellitus
TNFα	Tumour necrosis factor alpha
TTR	Tracer: tracee ratio
VAT	Visceral adipose tissue
V/V	Volume for volume
WHR	Waist hip ratio

Presentations and Publications Relating to this Thesis

Abstracts

Gibb F, Reynolds R, Phillips D, Andrew R, Walker B. In obese men, lower circulating androgens restrain generation of oestrogens by aromatase, with adverse metabolic effects. British Endocrine Society 2008.

Natalie ZM Homer, Diego F Cobice, **Fraser W Gibb**, Gregorio G Naredo, Scott G Denham, Brian R Walker, Ruth Andrew. Derivatisation of estrogens enhances sensitivity of analysis by Liquid Chromatography Tandem Mass Spectrometry. British Endocrine Society 2011.

Fraser W Gibb, Kerry J McInnes, Ruth Andrew, Brian R Walker. Aromatase Inhibition in Healthy Men Induces Insulin Resistance, Elevated Blood Pressure, and Altered Plasma Lipids, with Limited Changes in Transcript Levels in Subcutaneous Adipose Tissue *Endocr Rev 32: P1-335*. ENDO 2011.

F W Gibb, R Andrew, M Dixon, C Clarke, BR Walker. Aromatase Inhibition in Post-Menopausal Breast Cancer Patients is Associated with Increased Body Fat and Insulin Resistance: A Case-Control Study. ENDO 2012.

Reviews

Gibb Fraser W., Strachan Mark W. J. Androgen deficiency and type 2 diabetes mellitus. *Clinical Biochemistry.* 2014; 47: 940 – 949.

Chapter 1

Introduction

Sex steroid hormones are thus named because of their well-established role in sexual development and fertility. Beyond their role in reproduction, considerable evidence supports effects upon body composition and, more recently, upon fuel metabolism and cardiometabolic risk (Mauvais-Jarvis *et al.* 2013). Phylogenetic analysis suggests the single ancestral receptor (ANcSR1), from which other steroid receptors evolved, was estrogen responsive and, in predating sexual reproduction, was likely to have had a role in regulating cellular metabolism (Eick & Thornton 2011). Compared to age-matched men, premenopausal women have greater insulin sensitivity when adjusted for lean mass (Park *et al.* 2003). Furthermore, evidence from multiple animal models supports a critical metabolic role for sex steroid hormones, including the protection from insulin resistance afforded to estradiol replete rodents (Stubbins *et al.* 2012). Conversely, menopause and ovariectomy result in adverse effects upon metabolic health. In men, subnormal testosterone levels are associated with increased visceral adiposity (Nielsen *et al.* 2007) as well as an increased risk of developing type 2 diabetes mellitus (T2DM) (Ding *et al.* 2006).

Whilst the testes and ovaries are the major source of androgens and estrogens, respectively, the adrenal glands are also responsible for androgen generation and adipose tissue is increasingly recognised as a significant source of sex steroid hormone production. Indeed, in men and postmenopausal women, estrogens are primarily generated in adipose tissue from the conversion of androgens, by the enzyme aromatase (Simpson *et al.* 1999) (figure 1.1). In this context, plasma estrogens, whilst perhaps reflecting adipose tissue generation (Belanger *et al.* 2006), do not primarily operate as hormones in the classical sense (*i.e.* by exerting their

effect at a site distant from their generation) (Simpson *et al.* 2005). The importance of this local adipose hormone generation, which also involves glucocorticoid and androgen metabolism, places limits upon the usefulness of studying plasma hormone levels and, equally, in relying upon systemic administration of sex hormones to assess their adipose effects. Indeed, the steroid content of adipose tissue has been estimated to be between 40 to 400 fold greater than that of plasma (Belanger *et al.* 2002). The term 'intracrinology' has been coined to encompass this important process of local hormone generation and action. Pharmacological inhibition of aromatase is known to markedly suppress circulating estrogens and, in men and post-menopausal women, this is presumably a consequence of action in the adipose compartment.

A significant body of observational and mechanistic evidence supports a role for sex steroid hormones in relation to body composition, insulin sensitivity, diabetes risk and mortality. This thesis will examine the influence of sex hormones upon insulin sensitivity, adipose tissue and body composition, principally through assessment of the effects of pharmacological aromatase inhibition in both men and women.

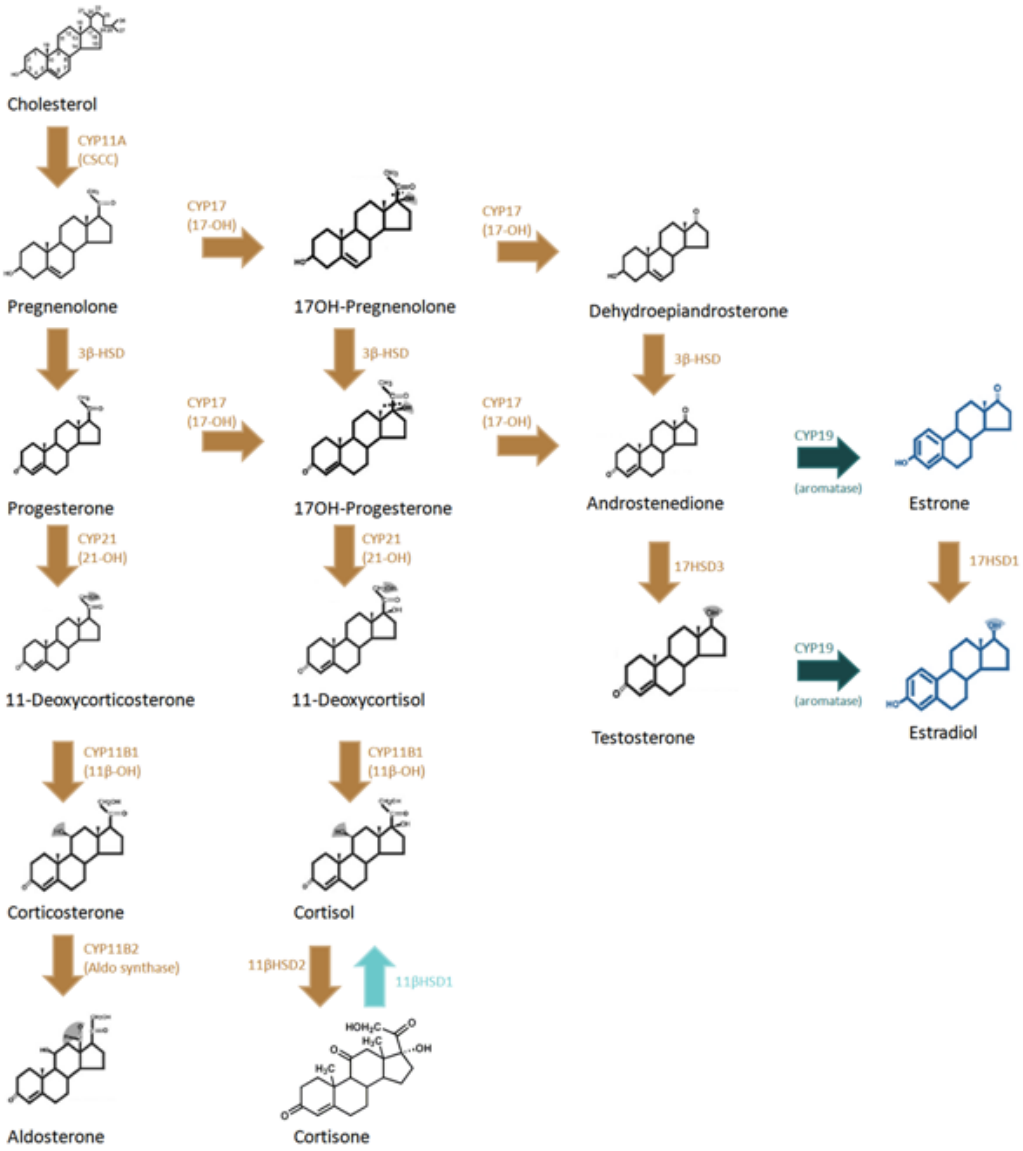


Figure 1.1 Steroid hormone biosynthetic pathway (adapted from Payne & Hales *et al.* 2004)

1.1 Sex steroids and body composition

1.1.1 Visceral and subcutaneous adipose tissue

The increasing incidence of type-two diabetes, and its attendant cardiovascular complications, is largely a result of rising levels of obesity. Risk of cardio-metabolic disease varies widely within the obese population, particularly as a function of body fat distribution. Adipose tissue distribution is conventionally described as either android (central, abdominal, male pattern) or gynoid (peripheral, gluteo-femoral, female pattern). Android obesity is a much stronger risk factor for the development of diabetes and cardiovascular disease than is gynoid obesity. Following menopause women typically shift from a gynoid to android body habitus (Kuk *et al.* 2005), with a coincident convergence in cardiovascular and diabetes risk with reference to their male peers (Lemieux *et al.* 1994). It is this interesting observation from nature which initially stimulated interest in the contribution of sex steroid hormones (particularly estrogens, which decline precipitously after menopause) to control of body fat distribution. Subsequently, humans with vanishingly rare genetic mutations controlling sex steroid hormone generation or action and elegant transgenic animal models have lent further support to the centrality of estrogens and androgens in the control of adipose distribution.

A substantial body of evidence has accumulated in support of the proposition that central fat distribution, and in particular visceral fat, plays a role in the development of cardio-metabolic disease (Wajchenberg 2000). The INTERHEART study demonstrated, in a cohort of 27,098 men and women, that Waist-hip ratio (WHR) (a proxy for central adiposity) was a more accurate predictor of incident myocardial

infarction than body mass index (BMI) (a proxy for generalised obesity) (Yusuf *et al*. 2005). A number of studies have sought to determine the differential effects of visceral and subcutaneous adipose tissue upon insulin sensitivity, all reaching broadly similar conclusions. Utilising computed tomography to quantify subcutaneous and visceral fat depots permitted the observation that 54% of the variance in insulin sensitivity, in men and women, can be accounted for by intra-abdominal fat volume (Cnop *et al*. 2002), congruent with earlier observations limited to post-menopausal women (Brochu *et al*. 2000). Further support for this hypothesis is offered by the observation that insulin sensitive obese post-menopausal women have 49% less visceral adipose mass than their insulin resistant peers (Brochu *et al*. 2001). In addition to the deleterious effects of visceral fat, some evidence exists to support a metabolically protective role for lower limb fat depots (Van Pelt *et al.* 2005).

Visceral adipose tissue typically accounts for between 5 and 20% of total adipose mass, yet appears to contribute disproportionately to the adverse outcomes associated with obesity. The visceral depot differs from subcutaneous fat not only in its unique anatomical relationship with the liver but also with respect to its transcriptomic and biochemical activity. The 'portal paradigm' seeks to explain the association of visceral fat with adverse metabolic outcomes by suggesting increased non-esterified fatty acid (NEFA) flux and inflammatory cytokines from visceral adipose tissue result in hepatic insulin resistance. Visceral adipose tissue contains a larger proportion of macrophages than subcutaneous fat, which may be associated with the production of atherogenic cytokines (Weisberg *et al.* 2003). Visceral adiposity is also associated with higher levels of plasminogen activator inhibitor-1 (PAI-1) and low-grade inflammation (Van Gaal *et al.* 2006). It remains a possibility that visceral fat is simply

a marker for generalised ectopic fat deposition; however surgical removal of intra-abdominal fat appears to mitigate insulin resistance in rodents (Gabriely *et al.* 2002) and humans (Thörne *et al.* 2002), thus lending support to a pathogenic role. Conversely, in mice, transplantation of subcutaneous fat into the visceral compartment of a recipient animal, results in improved insulin sensitivity and reduced fat mass (Tran *et al.* 2008).

Visceral adipose tissue is less sensitive to the anti-lipolytic effects of insulin (figure 1.2). It exhibits higher responsiveness to pro-lipolytic catecholamine β_1 and β_2-adrenoreceptor signalling and lower responsiveness to anti-lipolytic α_2-adrenoreceptor signalling, compared with subcutaneous fat (Lafontan *et al.* 2003). Adipocytokine production also demonstrates site specificities, with leptin secretion higher in subcutaneous adipocytes (van Harmelen *et al.* 1998) and adiponectin higher in visceral adipocytes.

Obesity results in increased basal lipolysis with larger fat cells and increased TNFα production (Arner *et al.* 2005). Whilst catecholamine-induced lipolysis is reduced in the subcutaneous depot, the opposite is true in the visceral compartment and this is more pronounced in men (Nielsen *et al.* 2004).

Assessment of transcript expression between visceral and subcutaneous adipocytes has shown that, as expected, leptin transcript levels are greater in subcutaneous adipocytes (Montague *et al.* 1998). No significant difference in the transcript levels for lipoprotein lipase, hormone sensitive lipase, peroxisome proliferator-activated receptor gamma (PPARγ) and tumour necrosis factor alpha (TNFα) were noted

between compartments. Microarray analysis in obese individuals has identified additional differentially expressed transcripts, though further work is required to elucidate their role (Linder *et al*. 2004).

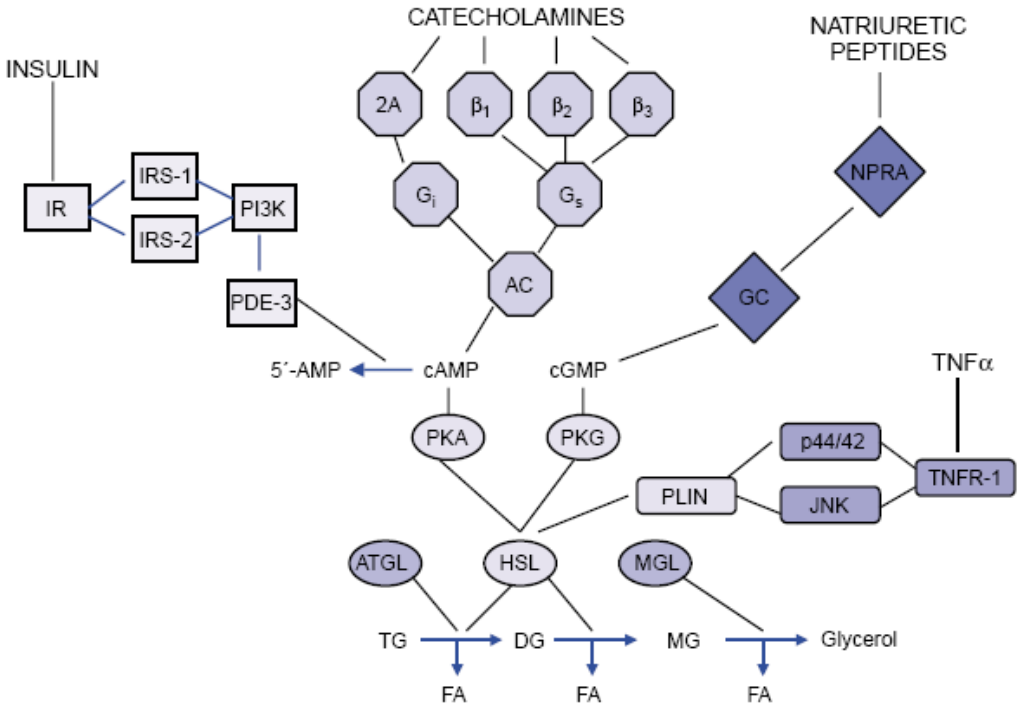


Figure 1.2 Regulation of lipolysis in human fat cells (from Arner *et al*. 2005)

β1,2,3, beta1,2,3-adrenergic receptors; α2A, α2A-adrenergicreceptor, Gi,s, inhibitory (i) or stimulatory (s) G-proteins; AC, adenylate cyclase; cAMP, cyclic AMP; cGMP, cyclic GMP, NPRA, natriuretic peptide receptor A; GC, guanylyl cyclase; PKG, cGMP-dependent protein kinase; PKA, protein kinase A; TNFα, tumour necrosis factor alpha; P44/42 and JNK, MAP kinase pathways; TNFR1, TNFα receptor 1;ATGL, adipose-tissue-specific triglyceride lipase; HSL, hormone-sensitive lipase; MGL,monoglyceride lipase; TG, triglycerides; DG, diglyceride; MG, monoglyceride; FA, fatty acid; IR, insulin receptor; IRS-1,2, insulin receptor substrates 1 and 2; PI3K, phosphadilyl inositol 3 kinase; PDE3, phosphodiesterase 3.

1.1.2 Direct effects of sex steroids on adipocyte function

1.1.2.1 Estrogens

Estrogens primarily mediate their effects through two nuclear receptors: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ); both of which exist in multiple isoforms (Mauvais-Jarvis *et al.* 2013). When bound, these receptors dissociate from their chaperone heat-shock protein, dimerize and bind to estrogen response elements (ERE) to influence transcription. Interestingly ERα appears to promote transcription, whilst ERβ may impede this process by forming heterodimers with ERα (Hall *et al.* 1999). In addition to their action as classical nuclear receptors, estrogen receptors also localise in caveolae where they can facilitate rapid signalling through activation of growth-factor receptors and G proteins, which may be of particular relevance in relation to the metabolic effects to estrogens (Hammes *et al.* 2007). No consensus exists with respect to the distribution of estrogen receptors in adipose tissue; both ERα and ERβ have been detected in mature adipocytes and whilst some investigators confirm a similar situation in preadipocytes (Pedersen *et al*. 2001), others have isolated only ERα (Dieudonné *et al*. 2004). Similarly conflicting reports have identified either ERα (Dieudonné *et al*. 2004) or, alternatively, ERβ1 (Pedersen *et al*. 2001) as the predominant receptor in adipose tissue. The expression of ERα is equal across depots and gender, however it is suggested that ERβ levels may be higher in women and in subcutaneous fat (Dieudonné *et al*. 2004). *In vitro,* administration of estradiol upregulates subcutaneous ERα and β expression in women, but only ERα expression in subcutaneous and visceral adipocytes in men (Pequery *et*

al. 2004). Menopausal status does not appear to influence the distribution of estrogen receptors (Shin *et al.* 2007).

Recent work has sought to define the effects of estrogens upon adipose transcription patterns (summarised in table 1.1). In ovariectomized mice, estradiol replacement down-regulated genes favouring lipid storage, including lipoprotein lipase (LPL), acetyl-CoA carboxylase-1 (ACC-1) and fatty acid synthase (FAS). Reductions in ACC-1 and FAS were attributed to down-regulation of liver X receptor α (LXRα) and SREBP-1c (D'Eon *et al.* 2005). In male mice, estradiol administration resulted in down-regulation of genes for monocyte-chemoattractant protein (MCP) and androgen receptor, in visceral and subcutaneous fat respectively (Shinozaki *et al.* 2007).

Gene	Estrogen	Effect	Androgen	Effect
Steroid hormone synthesis and metabolism				
HSD11B1 11βHSD1	**Human female**: Ten-fold increased mRNA expression in cultured omental pre-adipocytes (Dieudonne 2006) **Human male:** No effect on mRNA expression in cultured omental pre-adipocytes (Dieudonne 2006) **Human SGBS cells**: Increased mRNA expression through ERβ activation (McInnes 2012)	↑ → ↑	**Human male:** Increased mRNA expression in cultured omental pre-adipocytes (Dieudonne 2006)	↑
CYP19A1 Aromatase	**Human female**: 50% reduction in mRNA expression in cultured omental pre-adipocytes (Dieudonne 2006) **Human male:** 2.4 fold increased mRNA expression in cultured pre-adipocytes (Dieudonne 2006)	↓ ↑	**Human male:** Increased mRNA expression in cultured omental pre-adipocytes (Dieudonne 2006)	↑
Steroid hormone receptors				
ESR1 Estrogen receptor α	**Female ovariectomized mouse**: No change in perirenal adipose mRNA expression in E2 treated animals (D'Eon 2005) **Human female:** Increased mRNA expression in adipocytes from subcutaneous but not abdominal compartment (Dieudonne 2004) **Human male:** Increased mRNA expression in adipocytes from subcutaneous and abdominal compartment (Dieudonne 2004)	→ ↑→ ↑		

Gene	Estrogen Text	Effect	Androgen	Effect
ESR2 Estrogen receptor β	**Human female:** Increased mRNA expression in cultured adipocytes from subcutaneous but not abdominal compartment (Dieudonne 2004) **Human male:** No effect on mRNA expression in subcutaneous and abdominal cultured adipocytes (Dieudonne 2004)	↑ ➔		
Adipogenesis, lipogenesis and lipolysis				
FASN Fatty acid synthase	**Female ovariectomized mouse**: Reduced perirenal adipose mRNA expression in E2 treated animals (D'Eon 2005)	↓	**Female ovariectomized mouse**: Increased mRNA expression in visceral adipose tissue following DHT treatment (McInnes 2006)	↑
SREBF1 Sterol regulatory element binding transcription factor 1c	**Female ovariectomized mouse**: Reduced perirenal adipose mRNA expression in E2 treated animals (D'Eon 2005)	↓		
SREBF2 Sterol regulatory element binding transcription factor 2			**Female ovariectomized mouse**: Increased mRNA expression in visceral adipose tissue following DHT treatment (McInnes 2006)	↑
NR1H3 Liver X receptor α	**Female ovariectomized mouse**: Reduced perirenal adipose mRNA expression in E2 treated animals (D'Eon 2005)	↓		

Gene	Estrogen	Effect	Androgen	Effect
LPL Lipoprotein lipase	**Human female**: Increased protein expression in cultured sc adipocytes with higher dose estradiol (reduced at lower doses) (Palin 2003) **Female ovariectomized mouse**: Reduced perirenal adipose mRNA expression in E2 treated animals (D'Eon 2005)	↓ ↑ ↓	**Female ovariectomized mouse**: Increased mRNA expression in visceral adipose tissue following DHT treatment (McInnes 2006)	↑
LIPE Hormone sensitive lipase	**Human female**: Increased protein expression in cultured sc adipocytes with higher dose estradiol (Palin 2003) **Female ovariectomized mouse**: Unchanged perirenal adipose mRNA expression in E2 treated animals (D'Eon 2005) **Human female (post-menopausal)**: No change in protein expression or mRNA in sc adipose tissue with estradiol therapy (Zang 2007)	↑ → →	**Female ovariectomized Cynomolgus Monkey**: Decreased visceral adipose mRNA expression in DHT treated animals (Nantermet 2008) **Human female (post-menopausal)**: Reduced protein expression in sc adipose tissue but no change in mRNA with testosterone therapy (Zang 2007) **Human subcutaneous cultured pre-adipocytes**: Decreased protein expression in testosterone treated cells (Dicker 2004)	↓ ↓ ↓
PPARG Peroxisome Proliferator-activated receptor gamma	**Female ovariectomized mouse**: No change in perirenal adipose mRNA expression in E2 treated animals (D'Eon 2005)	→	**Female ovariectomized mouse**: Increased mRNA expression in visceral adipose tissue following DHT treatment (McInnes 2006) **Human mesenchymal stem cell derived adipocytes**: Dose dependent reduced mRNA expression following incubation with DHT (Gupta 2008) **Female ovariectomized Cynomolgus Monkey**: Decreased visceral adipose mRNA expression in DHT treated animals (Nantermet 2008)	↑ ↓ ↓

Gene	Estrogen	Effect	Androgen	Effect
ADRA2A α-2-adrenergic receptor	**Human female**: Increased mRNA expression in subcutaneous adipose tissue and cultured subcutaneous adipocytes (Pedersen 2004)	↑		
ADRB2 β2-adrenergic receptor			**Human subcutaneous cultured pre-adipocytes**: Decreased protein expression in testosterone treated cells (Dicker 2004)	↓
ACACA Acetyl Co-A carboxylase	**Female ovariectomized mouse**: Reduced perirenal adipose mRNA expression in E2 treated animals (D'Eon 2005)	↓	**Human mesenchymal stem cell derived adipocytes**: Dose dependent reduced mRNA expression following incubation with DHT (Gupta 2008)	↓
UCP2 Uncoupling protein 2	**Female ovariectomized rat:** Increased white adipose tissue mRNA expression in E2 treated animals (Pedersen 2001)	↑		
DGAT2 Diacylglycerol O-acyltransferase 2			**Human mesenchymal stem cell derived adipocytes**: Dose dependent reduced mRNA expression following incubation with DHT (Gupta 2008) **Female ovariectomized Cynomolgus Monkey**: Decreased visceral adipose mRNA expression in DHT treated animals (Nantermet 2008)	↓ ↓
CTNNB1 β-catenin			**Female ovariectomized Cynomolgus Monkey**: Increased visceral adipose mRNA expression in DHT treated animals (Nantermet 2008)	↑
Lipid and sterol metabolism				
CETP Cholesterol ester transfer protein			**Female ovariectomized Cynomolgus Monkey**: Increased visceral adipose mRNA expression in DHT treated animals (Nantermet 2008)	↑

Gene	Estrogen	Effect	Androgen	Effect
Adipokines				
LEP Leptin	**Female ovariectomized rat**: Increased retroperitoneal adipose mRNA expression in E2 treated animals (Wu-Peng 1999) **Female ovariectomized mouse**: Increased perigonadal adipose mRNA expression in E2 treated animals (Gui 2004)	↑ ↑	**Female ovariectomized rat**: Decreased retroperitoneal adipose mRNA expression in testosterone treated animals (Wu-Peng 1999) **Male gonadectomized mouse**: Unchanged perigonadal adipose mRNA expression in DHT treated animals (Gui 2004) **Human mesenchymal stem cell derived adipocytes**: Dose dependent reduced mRNA expression following incubation with DHT (Gupta 2008)	↓ → ↓
ADIPOQ Adiponectin	**Female ovariectomized mouse**: Increased perigonadal adipose mRNA expression in E2 treated animals (Gui 2004)	↑	**Male gonadectomized mouse**: Unchanged perigonadal adipose mRNA expression in DHT treated animals (Gui 2004)	→
RETN Resistin	**Female ovariectomized mouse**: Decreased perigonadal adipose mRNA expression in E2 treated animals (Gui 2004) **Female ovariectomized rat**: Decreased adipose (multiple depots) mRNA expression in E2 treated animals (Huang 2004)	↓ ↓	**Male gonadectomized mouse**: Unchanged perigonadal adipose mRNA expression in DHT treated animals (Gui 2004)	→
RBP4 Retinol binding protein 4			**Female ovariectomized Cynomolgus Monkey**: Decreased visceral adipose mRNA expression in DHT treated animals (Nantermet 2008)	↓
Miscellaneous				
IGF1 Insulin-like growth factor-1			**Female ovariectomized Cynomolgus Monkey**: Increased visceral adipose mRNA expression in DHT treated animals (Nantermet 2008)	↑

Table 1.1 Effects of estrogens and androgens upon mRNA transcript ± protein levels in adipose tissue

Administration of estradiol to rats resulted in a marked reduction in LPL activity (Hamosh *et al.* 1975), which is consistent with the finding in cell culture studies of reduced LPL mRNA when incubated with estradiol (Homma *et al.* 2000); a unique estrogen response element on the LPL promoter has been identified which helps account for these findings (Homma *et al.* 2000). In ovariectomized rats, basal and isoproterenol stimulated lipolysis were reduced to 50% and 25%, respectively, in parametrial fat pads; these changes were reversed with exogenous estradiol therapy (Darimont *et al.* 1997). In human female subcutaneous adipocytes, estradiol reduced LPL activity whilst increasing hormone sensitive lipase (HSL) activity and glycerol release (as a proxy of lipolysis) (Palin *et al.* 2003).

The demonstration that estradiol directly increases α2A-adrenoreceptors in subcutaneous adipocytes (but not in visceral adipocytes) provides an appealing mechanistic link between higher estrogen levels and gynoid fat distribution. Increased α2A-adrenoreceptor expression results in attenuated epinephrine induced lipolysis and favours subcutaneous fat deposition (Pedersen *et al.* 2004).

Estrogens also play a role in adipose development, having been shown as early as the 1970s to stimulate preadipocyte proliferation (Roncari *et al.* 1978). More recently human subcutaneous and visceral preadipocyte proliferation was demonstrated to a greater extent in women than men, following *in vitro* incubation with estradiol (Anderson *et al.* 2001). Whether estrogens are capable of inducing adipocyte differentiation remains more controversial (Cooke *et al.* 2004). However the increase in fat cell number observed in models of estrogen deficiency, particularly in the visceral compartment, does suggest a fundamental role for estrogens in this regard.

Animal models of estrogen deficiency provide further evidence of the centrality of estrogens in adipocyte function. Ovariectomized mice increase adipose mass and adipocyte size in excess of their pair-fed littermates. Estradiol replacement ameliorates this phenotype by reducing lipogenic gene expression, increasing lipolysis and up-regulating skeletal muscle genes involved in fatty acid oxidation (D'Eon *et al.*, 2005). Male estrogen receptor α knockout (ERαKO) mice display a distinct phenotype of insulin resistance with adipocyte hypertrophy and hyperplasia (Heine *et al.* 2000). In female ERαKO mice, obesity is observed in the context of a ten-fold elevation in estradiol levels; which may be relevant as ERβ is unaffected in this model. When ovariectomy was performed, a reduction in body weight (6%) was accompanied by a 45% reduction in fat pad mass and a 16% reduction in adipocyte size; thus suggesting ERβ mediates deleterious effects upon adipose tissue (Naaz *et al.* 2002).

1.1.2.2 Androgens

Androgens exert their biological effects when bound to the androgen receptor (AR) which, like the estrogen receptor, is a member of the nuclear hormone receptor family. Bound receptors form homodimers which interact with androgen response elements to effect transcription. As is the case with estrogens, alternative non-genomic signalling pathways are likely to exist and this is an area of active research.

In humans, 60% of circulating testosterone is avidly bound to sex hormone binding-globulin, approximately 38% is weakly bound to albumin and the remainder

circulates in a 'free' state. DHT is the most potent activator of AR and is generated from testosterone by 5α-reductase.

Androgen receptors have been identified in adipocytes and preadipocytes from both men and women. Androgen receptors are more plentiful in visceral than subcutaneous preadipocytes in both sexes. AR expression decreases during the differentiation process and has been shown to increase following dihydrotestosterone (DHT) exposure (Dieudonne *et al.* 1998), although others have demonstrated down-regulation in similar circumstances (Anderson *et al.* 2002). The effects of androgens upon adipose tissue mRNA transcript levels are summarised in table 1.1.

Employing a serial analysis of gene expression (SAGE) approach, short term ante-mortem exposure of male mice retroperitonal adipose tissue to DHT was shown to stimulate genes involved in glycolysis, fatty acid synthesis, triacylglycerol production, cell proliferation and differentiation (Bolduc *et al.* 2004). However, the same authors demonstrated quite distinct effects upon longer term exposure to DHT, including promotion of lipid utilization, inhibition of lipogenesis, increased adipocyte apoptosis, increased adiponectin C1Q, increased estrogen clearance and elevated LPL expression (Bolduc *et al.* 2007). Recently, ovariectomized cynomolgus monkeys were treated with DHT and gene expression assessed, in visceral and subcutaneous adipose, by microarray analysis and real-time polymerase chain reaction (PCR). Transcription in visceral and subcutaneous depots was strongly correlated ($r=0.78$, $p<0.001$). Significant effects were noted in genes responsible for extra-cellular matrix, cell adhesion and cytoskeletal structure. Consistent down-regulation of genes

involved in fatty acid, phospholipid and triglyceride metabolism was noted, including HSL (Nantermet *et al.* 2008).

In human female subcutaneous adipocytes, incubation with DHT increased LPL expression whilst inhibiting that of HSL (the latter effect apparently independent of AR activation) (Anderson *et al.* 2002). Testosterone was shown to increase LPL activity in young men, but only when co-administered with an aromatase inhibitor (suggesting that estradiol, arising from androgen aromatization, negates this process) (Zmuda *et al.* 1993).

In men and women, DHT effects a 50% reduction in epinephrine stimulated lipolysis in preadipocytes from the subcutaneous, but not visceral, compartment when studied *in vitro* (Dicker *et al.* 2004); this is associated with a 50% reduction in HSL expression and attenuated expression of β2-adrenoreceptors. Postmenopausal women, treated with a 3-month course of testosterone, down-regulated abdominal subcutaneous fat expression of HSL and phosphodiesterase-3B (PDE-3B) in concert with demonstrable reductions in lipolysis (Zang *et al.* 2007). *In vitro* testosterone also appears to mediate increased insulin resistance in female subcutaneous abdominal adipocytes (Corbould 2007).

Insights from genetically modified models highlight the importance of androgen signalling with respect to metabolism. AR null mice develop late onset obesity and are less physically dynamic with reduced oxygen consumption. However, food intake is unchanged and insulin resistance is not typical. AR null mice display down-regulation of HSL and thermogenic uncoupling protein 1 and up-regulation of PPARγ

and adiponectin in adipose tissue (Fan *et al*. 2005; Sato *et al.* 2003). In orchidectomized mice, DHT replacement but not estradiol, resulted in obesity, reduced energy expenditure, reduced fat oxidation but without effects on food consumption or locomotor activity. DHT replacement was associated with increased plasma HDL cholesterol and triglyceride levels (Movérare-Skrtic *et al.* 2006). In ovariectomized mice, DHT replacement resulted in increased body weight and visceral fat mass. In addition, DHT effected up-regulation of FAS, SREBP-2 and LPL and a reduction in AMPK phosphorylation in visceral fat (McInnes *et al.* 2006). This study in particular confers greater import upon altered ratios of AR:ER activation as a putative mediator of adverse metabolic sequelae.

Androgens appear to play a negative role in the regulation of adipocyte development. DHT has been shown to reduce adipogenesis in pluripotent stem cells and promote development along a myogenic line (Singh *et al*. 2003; Singh *et al.* 2006). Site specific anti-adipogenic effects were noted in rat preadipocytes exposed to DHT (Dieudonne *et al.* 2000), whilst some *in vitro* studies in human preadipocytes have failed to demonstrate any effect (Dicker *et al.* 2004; Corbould *et al.* 2007).

1.1.3 Energy intake and expenditure: sex steroid effects

In addition to direct effects upon adipocyte function, sex steroids are known to influence energy intake and expenditure. Ovariectomy leads to weight gain in rodents, which is prevented by estrogen replacement. Increased food intake is ameliorated by estrogen replacement, which increases excitatory POMC inputs to the arcuate nucleus in mice, independent of leptin (Gao *et al.* 2006). Energy expenditure

is reduced by 11% in ERα knockout mice with no significant difference in food intake (Heine *et al.* 2000), with little effect observed in ERβ knockout mice. Exogenous estradiol administration is also associated with increased sensitivity to leptin in male and female rats (Clegg *et al*. 2006). Estradiol administration may also reduce hypothalamic expression of the anabolic peptide NPY, as suggested by variability across the estrus cycle in mice (Olofsson *et al.* 2009). The role of androgens in modulating appetite and energy expenditure is much less well defined and, as is often the case, complicated by the confounding effect of changes in estrogen concentration when aromatizable androgens are administered. Gender differences in food intake may originate from androgen programming effects on POMC neurons in the arcuate nucleus during early development, as suggested in a murine model (Nohara *et al.* 2011).

1.1.4 Sex steroid effects upon body composition: observational evidence

1.1.4.1 Estrogens (women)

The direct effect of menopause upon body composition is controversial and it has been suggested that many of the observed changes are primarily a consequence of ageing, however the balance of evidence supports a significant effect of menopausal status upon WHR and fat mass (Poehlman *et al.* 2002). In women, a number of observational studies have investigated the relationship between menopausal status and adipose metabolism with varying results. In pre- and postmenopausal women,

matched for visceral adipose area, no difference in subcutaneous abdominal and femoral adipose LPL activity or epinephrine-induced lipolysis was noted (Mauriege *et al.* 2000). Similar investigations have elicited significantly lower basal lipolysis in gluteal subcutaneous adipose tissue, with elevated LPL activity in abdominal and gluteal subcutaneous fat, in postmenopausal women (Ferrera *et al.* 2002). Another study, comparing pre- and postmenopausal women, suggested larger omental fat cell size, higher omental basal lipolysis and higher omental/subcutaneous (om/sc) LPL activity in the latter group. However, with the exception of om/sc LPL activity, menopausal status was not independently associated with these variables when corrected for visceral adipose tissue area (Tchernof *et al.* 2004). Previously in obese women, plasma estradiol levels were negatively correlated, and total testosterone positively correlated, with total post-heparin LPL activity (Iverius *et al.* 1988).

1.1.4.2 Androgens (women)

Fewer studies have investigated the association of circulating androgens and obesity in women. In women with oligomenorrhoea or hirsutism, free androgen index (FAI) and testosterone levels were noted to be significantly greater in the obese (Taponen *et al.* 2003). A negative association has been observed between plasma testosterone levels and visceral fat in premenopausal obese women (Armellini *et al.* 1994).

1.1.4.3 Estrogens (men)

In men the influence of estrogens on obesity has not been extensively investigated. Plasma estradiol has been positively correlated with subcutaneous fat mass in healthy

young men (Nielsen *et al.* 2007), whilst other studies have failed to confirm this (Abate *et al.* 2002). Plasma estrone (which was closely correlated with subcutaneous adipose tissue levels) has been positively correlated with waist circumference and body mass index (BMI) in young men (Belanger *et al.* 2006).

1.1.4.4 Androgens (men)

A number of observational studies have been designed to investigate the relationship between androgens and obesity in men. Free plasma testosterone was inversely related to visceral adipose mass in young men as determined by magnetic resonance imaging (MRI) (Nielsen *et al.* 2007). This finding has been replicated by other investigators (Seidell *et al.* 1990) but not in a similar study, performed in a slightly older cohort, where the only inverse association was with subcutaneous fat (Abate *et al.* 2002). Tissue levels of DHT and testosterone also correlated negatively with waist circumference and BMI in young men (Bélanger *et al*. 2006). Waist circumference appears to be a stronger predictor of testosterone levels than BMI based on an analysis of 1548 men in the Tromsø study (Svartberg *et al.* 2004).

1.1.5 Effect of sex hormone replacement upon body composition

1.1.5.1 Women

Studies in which exogenous estrogens are administered to females are plentiful as a result of 'hormone replacement therapy' (HRT). However HRT studies rarely, if ever, provide pure insights into the effect of estrogens on adipose tissue for the

following reasons: progestins, with varying degrees of androgenicity, are often prescribed concurrently; a variety of different estrogen preparations are available; and pharmacological doses are required to overcome first pass hepatic metabolism, with largely deleterious effects. Specifically, exogenous oral estrogens act on the liver to modulate lipid metabolism and haemostatic factors. This has prompted the development of alternative delivery routes (*e.g.* percutaneous), which seek to obviate these unwanted effects (Turgeon *et al.* 2006). All these factors contribute towards the heterogeneity of 'HRT', which precludes any simplistic interpretation of this body of evidence. In addition to the heterogeneity of treatment options, good evidence exists to suggest age at treatment (and time from menopause) may be critical in determining the anti-atherogenic response (Mikkola *et al.* 2002); it is feasible that a similar temporal relationship may apply to adipose responsiveness.

HRT consisting of estradiol valerate and, perhaps significantly, the antiandrogenic progestin cyproterone acetate, resulted in sparing of the shift to androgenic fat distribution which was noted in early menopause age matched controls (Gambacciani *et al.* 1997). Similarly a cross-sectional study involving 2175 women confirmed the preservation of premenopausal fat distribution in women treated with a variety of HRT preparations, in recent onset menopause (Genazzini *et al.* 2006). A prospective study of estradiol and norethisterone in 38 postmenopausal women (mean age 53) also demonstrated significant reductions in android obesity (Arabi *et al.* 2003). Combined estrogen-progesterone HRT, in early postmenopausal women, prevented abdominal fat gain, as assessed by DEXA, in a randomised trial (Haarbo *et al.* 1991). However, after adjusting for confounding variables, no significant difference in either BMI or WHR was observed in a cross-sectional study investigating HRT effect in 671

women (Kritz-Silverstein *et al.* 1996). More detailed analysis of body fat distribution, with computed tomography, failed to show any difference in visceral or subcutaneous fat area between women on HRT (estrogen alone or estrogen-progesterone) and age and BMI matched controls (Ryan *et al.* 2002).

To assess the short term effects of estrogens upon fat metabolism, the response of whole body and subcutaneous adipose lipolysis to intravenous conjugated estrogens was assessed. Estrogen was noted to decrease subcutaneous fat basal lipolysis but did not affect whole body lipolysis or insulin mediated suppression (Van Pelt *et al.* 2006).

The only study investigating the metabolic effects of estrogen antagonism (partial agonism) was performed in breast cancer patients receiving tamoxifen, which was shown to promote the accumulation of visceral and hepatic fat (Nguyen *et al*., 2001).

Investigating the potential metabolic benefits of androgen replacement in postmenopausal women is a relatively recent concept, originating from prior observations of improved energy levels, sexual function and quality of life. Three months of testosterone replacement (or testosterone and estradiol in combination) to women with a mean age of 55 years resulted in increased body weight and lean mass but not fat mass (Zang *et al.* 2006). Postmenopausal women receiving a 9 month course of nandrolone (a weakly androgenic anabolic steroid) lost subcutaneous fat whilst gaining fat in the visceral compartment (Lovejoy *et al.* 1996). DHEA supplementation in elderly women over a 2-year period did not alter body composition (Nair *et al.* 2006).

Assessing the effects of hormonal treatment in female to male transsexuals affords a rare insight into the consequences of androgen therapy in genetic females. Testosterone, administered on a fortnightly basis, resulted in reductions in subcutaneous fat and a concurrent increase in visceral depots (Elbers *et al.* 2003). This corroborated earlier findings of reduced subcutaneous and increased visceral fat occurring alongside increased thigh muscle area, as assessed by MRI (Elbers *et al.* 1999a). In female to male transsexuals, gluteal and abdominal subcutaneous adipocytes are rendered smaller by testosterone administration and basal lipolysis is increased in the latter depot (although no differences in response to isoproterenol or insulin were noted) (Elbers *et al.* 1999b).

Androgen excess is implicated in the pathogenesis of polycystic ovary syndrome (PCOS), which is associated with obesity, insulin resistance and elevated cardiovascular risk. In this context, the androgen receptor antagonist flutamide effects reductions in visceral fat (Gambineri *et al.* 2004; Gambineri *et al.* 2006). The success of this strategy in PCOS raises the possibility of utility in the postmenopausal context, which is also characterized by an elevated androgen to estrogen ratio. However 9 months of spironolactone (a mineralocorticoid and androgen receptor antagonist) therapy failed to alter body fat distribution (Lovejoy *et al.* 1996).

1.1.5.2 Men

Where female to male transsexuals offer insights into the effects of testosterone in women, male to female transsexuals provide a unique opportunity to investigate the effects of estrogen therapy (in combination with androgen antagonism) in men. In

this setting, 20 non-obese men received daily ethinyl estradiol and cyproterone acetate, which effected increases in visceral and subcutaneous fat of 18% and 38%, respectively. Beneficial changes in HDL cholesterol, LDL cholesterol and hepatic lipase activity were counterbalanced by elevations in blood pressure, triglyceride and reductions in LDL particle size (Elbers *et al.* 2003). These changes in body composition are in accord with earlier observations by the same authors (Elbers *et al.* 1999a). Following a year of oestrogen and anti-androgen therapy, subcutaneous fat cell size increased as did basal lipolytic rate (though response to insulin and isoproterenol was unchanged) (Elbers *et al*. 1999b).

Akin to estrogen replacement trials in women, androgen replacement is the most investigated hormonal manipulation in men. Suppressing endogenous hormone release with GnRH, in healthy young men, afforded the opportunity to assess the dose response effect of testosterone replacement on fat distribution (determined by MRI). Subphysiological replacement caused gains in inter-muscular, intra-abdominal and, in particular, subcutaneous adipose tissue. Higher testosterone doses did not influence intra-abdominal fat but did reduce subcutaneous and, most markedly, inter-muscular fat (Woodhouse *et al.* 2004). In young men, six weeks of exogenous testosterone resulted in reduced LPL activity and an increased lipolytic response to norepinephrine in abdominal, but not femoral or gluteal subcutaneous adipose tissue, with an accompanying reduction noted in WHR (Rebuffe-Scrive *et al.* 1991). The effect of one year of transdermal testosterone patches upon fat distribution was investigated in an older cohort of men (age >55 years) with symptomatic androgen deficiency and low-normal testosterone levels. An average increase in plasma testosterone of 30% was achieved, which effected a significant reduction in visceral fat, without changes

in subcutaneous or total body fat. Fat free mass and skeletal muscle mass increased with testosterone, whilst lipids, insulin and glucose levels were unaltered (Allan *et al.* 2007).

A short-term placebo-controlled trial of Testogel (n=183 active and n=179 placebo) in men with late onset hypogonadism (LOH), demonstrated approximately 1kg loss of fat mass with accompanying increases in lean mass at 6 months (Behre *et al.* 2012). Intra-muscular testosterone enanthate was associated with improvements in lean body mass and reductions in fat mass in older men (mean age 71) after a three year treatment period, in a placebo controlled study (Page *et al.* 2005).

Some of the effects of testosterone administration may be attributed to estradiol, produced from testosterone, by the action of aromatase in adipose tissue. This significant confounder can be addressed by the use of non-aromatizable androgens, such as DHT and oxandrolone. Oxandrolone was investigated in healthy elderly men, where a 12 week course was shown to reduce visceral and subcutaneous adipose tissue and reduce the VAT:SAT ratio; an effect observed up to 12 weeks after cessation of treatment (Schroeder *et al.* 2004).

Not all studies confirm the metabolic advantages of testosterone replacement. In 27 elderly men, with low bioavailable testosterone, testosterone replacement had no effect upon body composition, albeit increasing the average level of bioavailable testosterone by a modest 1.1 nmol/l (Nair *et al.* 2006). In hypogonadal men with T2DM, 40 weeks of intramuscular testosterone undecaonate resulted in increased lean

mass and reductions in fat mass, although this was limited to subcutaneous (but not visceral) abdominal adipose tissue volume (Gianatti *et al.* 2014).

Androgen deprivation therapy (ADT) in prostate cancer provides an opportunistic means to assess the effects of testosterone in determining body composition and appears to increase fat mass at the expense of lean mass (Smith *et al*. 2004). A prospective observational study in 26 men commenced on ADT demonstrated an average 13% increase in subcutaneous adipose area with a 23% increase in visceral adipose area (Hamilton *et al.* 2011).

When considering the consequences of androgen deficiency, it is important to recognise that levels of estrogens and androgens are inextricably linked by virtue of their common biosynthetic pathways. Across most relevant target tissues, both estrogens and androgens are capable of exerting effects, sometimes complementary, sometimes divergent. An elegant study in healthy volunteers, sought to untangle the differential contribution of estrogens and androgens with respect to symptoms and end-organ effects by suppressing endogenous sex steroids (with the GnRH analogue goserelin) and manipulating testosterone and estradiol levels with variable doses of testosterone gel and aromatase inhibition. Testosterone deficiency resulted in reduced lean mass and muscle strength, whereas estradiol deficiency was associated with increasing fat mass; both estradiol and testosterone deficiency independently contributed towards sexual dysfunction (Finkelstein *et al.* 2012). Whilst the clinical focus is often on recognising and treating androgen deficiency, many of the beneficial effects of normalising testosterone may be mediated, at least in part, by consequent normalisation of estradiol. Testosterone is converted to the more potent DHT by 5α-

reductase (5α-R), which amplifies androgen receptor activation. Treatment with the 5α-R inhibitor dutasteride (with concomitant testosterone replacement), did not significantly alter fat free mass, muscle strength, sexual function or prostate volume in healthy men, suggesting testosterone action is sufficient (Bhasin *et al.* 2012).

1.1.6 Methods for measuring body composition

A range of modalities are available for the assessment of human body composition, ranging from relatively straightforward anthropometric measurements, such as BMI and WHR, to complex techniques, such as underwater weighing (often considered the gold standard), dual-energy X-ray absorptiometry (DEXA) and isotope tracer methods. Body composition can be reduced to a 2-compartment model (fat mass and fat free mass) or a 4-compartment model, where FFM is subdivided into muscle, bone and water. All methods rely on assumptions with respect to the composition of compartments, which do not necessarily hold, particularly in certain disease states (Duren 2008).

1.1.6.1 Indirect methods

BMI is an easy to obtain clinical parameter with strong associations in relation to both morbidity and mortality. Interpretation must take into account racial differences and also recognise the limits in subjects where fat free mass contributes disproportionately to weight. As noted earlier, WHR appears to be a more discriminatory predictor of cardiovascular disease than BMI (Yusuf *et al.* 2005).

Measurement of skinfold thickness, by use of callipers across a range of anatomical locations, provides a more detailed assessment of subcutaneous fat content. Regression equations can be employed to estimate body fat percentage, based on skinfold thickness, with reasonable agreement reported with under-water weighing (Durnin and Wormersley 1974). However, this method is highly operator dependent and technically difficult in obese individuals, where it is much less well validated (Duren 2008).

Bioelectric impedance analysis (BIA) estimates body composition by measuring the resistance of the body to a small alternating electrical current. The impedance index (height^2/ resistance) is directly proportional to body water and regression equations are employed to provide an estimate of body fat percentage. The applicability of BIA results is strongly related to how closely subjects match the reference populations used to create the regression equations; consequently, the assumptions underlying this technique are not necessarily valid in obese individuals (Gray 1989).

1.1.6.2 Direct methods

Total body water can be measured by isotope dilution, where labelled water (most commonly deuterated) is administered and, following equilibration, the proportion of labelled to unlabelled water permits calculation of total body water. This technique is rendered expensive by the necessity for mass spectrometric analysis and, like other methods, depends on assumptions regarding the water content of fat free mass. Isotope dilution is prone to overestimate body fat, perhaps due to an inadequate time allowance for equilibration (Fogelholm 1997). Total body potassium is a less

commonly employed method where gamma radiation from the naturally occurring potassium isotope ^{40}K is measured. ^{40}K is present at a constant concentration which permits estimation of total body potassium and, by extrapolation, fat free mass. This technique relies on assumptions regarding the compartmental distribution of potassium and also the potassium content of fat free mass; it has largely been supplanted by other methods.

Underwater weighing (hydrodensitometry) is regarded as the gold standard for assessment of body composition. Measures of body weight, body volume and residual lung volume are incorporated in to either a two-compartment, or more recently multi-compartment models (including measures of bone density and total body water), to yield an estimate of body fatness. Air displacement plethysmography (ADP) obtains similar measurements (and results) to underwater weighing, and avoids the inconvenience associated with submersion (Fields *et al.* 2002).

Dual-energy x-ray absorptiometry relies on the differential attenuation of two low-energy levels passing through the body, to discriminate between tissue types. It has the advantage of providing information across different body sections (limbs and trunk) as well as quantification of bone mineral content. The process take no more than 20 minutes and is minimally invasive for subjects. Modern DEXA algorithms accord closely with underwater weighing, leading some to consider DEXA as a potential reference standard for measurement of body composition (Pritchard *et al.* 1993).

Whole body CT scanning can provide information on body fat content, based on the characteristic attenuation of adipose tissue. Image analysis software can produce estimates of body fat content. MRI imaging can provide similar information and avoids exposure to ionising radiation. Single slice CT imaging at the L4/5 level can provide specific information regarding the balance of visceral to subcutaneous adipose tissue.

1.2 Sex hormones and insulin sensitivity

Rodent models attest to the central role sex steroids play in regulating glucose metabolism. Ovariectomy induces obesity and insulin resistance in high fat diet fed mice; an effect which is prevented by physiological estradiol replacement (Stubbins *et al.* 2012). In male rats, gonadectomy induces an insulin resistant state, with marked reduction of skeletal muscle glucose uptake; testosterone replacement prevents development of this abnormal metabolic state (Holmäng *et al.* 1992).

1.2.1 Specific effects on muscle, liver and beta cells

In addition to adipose tissue effects, evidence has accumulated in support of a direct influence of sex steroid hormones across a range of insulin sensitive tissues, particularly liver and muscle. Sex steroids may also influence insulin secretion through effects upon beta cell function.

1.2.1.1 Muscle

In men, plasma testosterone concentration correlates positively with skeletal muscle expression of genes involved in oxidative phosphorylation (Pitteloud *et al.* 2005). GLUT4 expression is reduced in the skeletal muscle of testosterone deficient rats (Muthusamy *et al.* 2009).

Ovariectomy is associated with reduced skeletal muscle glucose uptake in female rats and is normalised by high does estradiol replacement. No effect on skeletal GLUT4 protein level was observed suggesting the mechanism may relate to downstream signalling or altered GLUT4 trafficking (Campbell *et al.* 2002). Other models assessing the effect of estrogen suggest a stimulatory effect of ERα upon GLUT4 expression in skeletal muscle (Barros *et al.* 2006), with ERβ stimulation perhaps responsible for the opposite effect (Barros *et al.* 2009). Not all animal models confirm a stimulatory effect on glucose uptake (Rogers *et al.* 2009). Whilst physiological levels of estradiol appear to promote insulin sensitivity, in rats supraphysiological levels exert the opposite effect, through repression of muscle GLUT4 (Barros *et al.* 2008). Estradiol increases skeletal muscle expression of genes involved in fatty acid oxidation in female mice (D'Eon *et al.* 2005) and also induces rapid phosphorylation of AMPK, a critical regulator of mitochondrial biogenesis and oxidative metabolism (Rogers *et al.* 2009).

1.2.1.2 Liver

ERα knockout mice display reduced suppression of hepatic glucose output during hyperinsulinaemic euglycaemic clamp studies, consistent with marked hepatic insulin resistance (Bryzgalova *et al.* 2006). High fat diet fed mice were protected from insulin resistance, at least in part through reduced hepatic triglyceride accumulation, with accompanying reductions in lipogenic transcript levels (Bryzgalova *et al.* 2008). Orchidectomy is associated with reduced insulin receptor protein levels and increased IRS-1 Serine$^{636/639}$ phosphorylation in rats, an effect which is reversed by both testosterone and estradiol (Muthusamy *et al.* 2011); abnormalities in hepatic glucose oxidation were reversed by testosterone (but not estradiol) replacement.

1.2.1.3 Beta cells

Testosterone has been shown to reduce beta cell apoptosis in a streptozotocin-induced diabetes in male rats through induction of anti-oxidant enzymes (Palomar-Morales *et al.* 2010). Pancreatic beta cells in high glucose culture medium are protected from apoptosis by testosterone, potentially through increased levels of survival proteins, sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA-2) and Bcl2 (Hanchang *et al.* 2013). In humans, T2DM is associated with increased beta cell endoplasmic reticulum stress, although whether testosterone sufficiency protects against this requires further investigation. Testosterone is also associated with increased insulin mRNA expression and insulin secretion, both in rats and primary culture of islet cells (Morimoto *et al.* 2001).

Estradiol is also associated with protective effects upon pancreatic beta cells, through interaction with extra-nuclear estrogen receptors, which exert their effect through modulation of cytosolic kinases. In rats, estradiol reduced beta cell dysfunction and lipid accumulation (Tiano *et al.* 2011) and, in mice, protects against apoptosis (Liu *et al.* 2009).

1.2.2 Adipokines

The relationship between adipokines and sex steroid hormones is complicated, and largely confounded by associated changes in adiposity, in models of androgen and estrogen deficiency. Short-term testosterone replacement in hypogonadal men was associated with reductions in circulating leptin and adiponectin (Kapoor *et al.* 2007). Transdermal estrogen replacement therapy reduced leptin and resistin, and increased adiponectin, in post-menopausal women (Chu *et al.* 2006). Pro-inflammatory cytokine levels rise in women following menopause (Pfeilschifter *et al.* 2002) and, in men, levels are inversely correlated with testosterone concentration (Kelly *et al.* 2013).

In adipose specific androgen receptor knock-out mice, elevated levels of retinol binding protein 4 (RBP4) have been observed (McInnes *et al.* 2012). RBP4 is associated with insulin resistance and metabolic syndrome in humans although levels are typically greater in men than in women.

1.2.3 Sex steroid effects upon insulin sensitivity: observational evidence in humans

1.2.3.1 Women

In a meta-analysis of sex hormones and type 2 diabetes risk, women with T2DM were shown to have significantly higher circulating estradiol levels, even after adjustment for BMI (Ding *et al*. 2006). However, no prospective evidence exists to support a causative role for estradiol in the development of diabetes. The same meta-analysis showed testosterone levels were significantly higher in women with T2DM, with a trend towards increased T2DM risk in prospective studies, in those with higher testosterone levels.

1.2.3.2 Men

T2DM and obesity are associated with an increased risk of testosterone deficiency but it is also true that testosterone deficiency likely carries an increased risk of developing T2DM and expanding body fat; these two related conditions are likely to have a bi-directional, mutually reinforcing relationship. Androgen levels consistently show negative correlations with insulin resistance, although it is often argued that this association is not independent of variability in body fat as is borne out by the majority of studies (Tchernof *et al.* 1995; Tsai *et al.* 2004; Pittleoud *et al.* 2005). Other studies failed to demonstrate any link between testosterone and insulin resistance (Nielsen *et al.*, 2007). A population based cohort study of middle-aged Finnish men (n = 651) identified baseline metabolic syndrome as a risk factor for incident testosterone

deficiency (2.6 fold increased risk which remained significant after correction for BMI or baseline testosterone) (Laaksonen *et al.* 2005).

Longitudinal observations from the Framingham Heart Study failed to detect an association between total testosterone and development of metabolic syndrome (Bhasin *et al.* 2011), however, elevated estrone (more so than estradiol) was associated with incident type 2 diabetes, even after correction for testosterone and other relevant confounders (Jasuja *et al.* 2013). Men with the lowest quartile of total testosterone had a significantly increased risk of incident metabolic syndrome (adjusted RR 1.38) in the Study of Health in Pomerania (Haring *et al.* 2009). Low testosterone may be particularly predictive of metabolic syndrome in non-obese men (Kupelian *et al.* 2006). Men with free testosterone in the lowest tertile are reported as being four times more likely to develop T2DM, independent of adiposity (Selvin *et al.* 2007); similar findings were noted in a meta-analysis of sex hormones and diabetes risk (Ding *et al.* 2006). Higher levels of estradiol have been associated with reduced cardiovascular risk in men aged over 56 years (Arnlöv *et al.* 2006) but also with an elevated risk of diabetes (Ding *et al.* 2006). Despite the consistency of association, these studies do not confirm causality. It is conceivable that lower testosterone in obese men may simply reflect greater androgen inactivation from an expanded adipose pool.

Androgen sensitivity may play a role in glucose homeostasis, as evidenced by the influence of CAG repeat length in the androgen receptor (associated with reduced AR sensitivity) upon insulin sensitivity (Mohlig *et al.* 2011).

1.2.4 Sex steroids and insulin sensitivity: interventional evidence in humans

1.2.4.1 Estrogens (women)

In addition to displaying no difference in basal carbohydrate and fat utilization, HRT treated women were significantly more insulin resistant, as determined by hyperinsulinemic euglycemic clamp studies (Ryan *et al.* 2002). This data conflicts with studies reporting reduced incidence of T2DM in HRT treated women (Margolis *et al.* 2004) and a crossover study involving 3 months estradiol treatment in diabetic postmenopausal women, which demonstrated improvements in HbA1c, C-peptide, fasting glucose and a non-significant trend towards higher glucose disposal rate (Andersson *et al.* 1997).

1.2.4.2 Androgens (women)

3 months of testosterone replacement (or testosterone and estradiol in combination; mean age 55) resulted in a 20% reduction in glucose disposal, along with increases in body weight and lean mass but not fat mass. Testosterone was also implicated in a reduction in HDL levels (Zang *et al.* 2006).

DHEA supplementation in elderly women over a 2-year period did not alter insulin sensitivity or body composition (Nair *et al* 2006).

In hypopituitary women of reproductive age, with proven androgen deficiency, testosterone replacement was shown to have, at worst, a neutral effect upon insulin resistance (Miller *et al*. 2007).

Assessing the effects of hormonal treatment in female to male transsexuals affords a rare insight into the consequences of androgen therapy in genetic females. Testosterone, administered on a fortnightly basis, resulted in deleterious alterations in lipids, including: reduced HDL cholesterol, reduced LDL size, increased triglycerides and increased hepatic lipase activity (Elbers *et al.* 2003).

The androgen receptor antagonist flutamide effects reductions in visceral fat, improves insulin sensitivity, reduces LDL cholesterol and elevates HDL (Gambineri *et al.* 2004; Gambineri *et al.* 2006) in women with PCOS. However, neither the anti-androgen spironolactone nor the weakly androgenic anabolic steroid nandrolone, altered glucose or insulin concentrations in post-menopausal women following 9 months of therapy (Lovejoy *et al.* 1996).

1.2.4.3 Androgens (men)

In young men with idiopathic hypogonadotrophic hypogonadism (mean age 40.8, n = 12), withdrawal of testosterone replacement resulted in increased insulin resistance (as determined by homeostatic model assessment – insulin resistance [HOMA-IR]) within two weeks, in the absence of any significant change in body composition, suggesting a direct role for sex steroid hormones in regulating insulin sensitivity in men (Yialamas *et al.* 2007). In older men, the non-aromatizable androgen,

oxandrolone, resulted in improved insulin sensitivity following 12 weeks of therapy (Schroeder *et al.* 2004).

TIMES2 was a randomised controlled trial involving 220 hypogonadal men with T2DM or metabolic syndrome; over a 12-month period, transdermal testosterone gel improved insulin sensitivity (16.4% as determined by HOMA-IR) as well as exerting beneficial effects upon LDL cholesterol and lipoprotein a (Jones *et al.* 2011). The protocol was subject to a high drop out rate and confounded by changes in other medications, so whilst a trend towards improved HbA1c was observed, this did not reach statistical significance. Similar effects have been observed in other small, short-term RCTs. A single-blinded study of 50mg transdermal testosterone in men with newly diagnosed T2DM (n=32) showed a significant improvement in HbA1c (mean -0.8%) and insulin sensitivity by HOMA-IR at one year, when compared with men receiving supervised diet and exercise advice only (Heufelder *et al.* 2009). Similar improvements were noted in a double-blind crossover study in 24 hypogonadal men with T2DM. After 3 months of intramuscular testosterone therapy, significant improvements in HOMA-IR, HbA1c (-0.37%) and fasting glucose were observed (Kapoor *et al.* 2006). Clearly larger studies, with longer follow-up, are required to confirm whether testosterone replacement should be considered as an adjunct to established diabetes therapies.

In contrast to these positive studies, in 27 elderly men, with low bioavailable testosterone, testosterone replacement had no effect upon insulin sensitivity (Nair *et al.* 2006). A study of elderly men with testosterone deficiency, who received testosterone patches for two years, failed to show any difference in a comprehensive

panel of insulin sensitivity measures, with respect to controls (Basu *et al.* 2007). Similarly, a randomised controlled trial of intramuscular testosterone undecanoate (1g 12 weekly over 40 weeks, n = 88) failed to demonstrate any improvement in either HbA1c or insulin sensitivity, as determined by HOMA-IR, in hypogonadal middle-aged men with T2DM (Gianatti *et al.* 2014).

Androgen deprivation therapy increased insulin resistance by 12%, as determined by HOMA-IR (Hamilton *et al*. 2011). Observational evidence, in older men with prostate cancer, shows GnRH agonist therapy to be associated with an increased risk of incident diabetes (adjusted HR 1.44) (Keating *et al.* 2006).

1.2.5 Methods for measuring insulin sensitivity

Insulin effects upon whole body glucose disposal are concentration-dependent and saturable, with the concentration required to achieve half-maximal response defining 'insulin sensitivity'. Insulin resistance typically reflects impairment of both glucose disposal and suppressibility of hepatic glucose production. A number of methodologies are available to quantify insulin sensitivity, either directly or indirectly (Muniyappa *et al.* 2007).

Hyperinsulinaemic euglycaemic clamp techniques, originally developed by DeFronzo and colleagues (DeFronzo *et al.* 1979), are generally considered the 'gold standard' method for measuring insulin sensitivity. Following an overnight fast, subjects receive a constant intravenous infusion of insulin, ultimately resulting in an elevated steady state insulin concentration. 20% dextrose solution is concurrently infused at a

variable rate (informed by measurement of arterialized blood glucose levels) to maintain euglycaemia. When steady state conditions are achieved, and assuming hyperinsulinaemia is sufficient to ablate hepatic glucose production, the glucose infusion rate (GIR) can be regarded as equal to the glucose disposal rate (M). M is often normalised to fat free mass, as skeletal muscle is the major source of insulin-mediated glucose uptake. Concomitant infusion of isotopically labelled glucose permits calculation of both hepatic glucose production and glucose disposal and addresses the potential problem of sub-maximal suppression of hepatic glucose production when attempting to assess glucose disposal. Glucose clamp studies are an accurate and direct means of assessing insulin sensitivity, however they are both time and labour intensive.

Another direct measure is the insulin suppression test, which utilises somatostatin analogues to suppress endogenous insulin secretion with concomitant constant infusions of glucose and insulin, to achieve steady state levels. Insulin suppression tests correlate well with clamp studies ($r = 0.93$) but, although easier to conduct, do not provide additional information on hepatic glucose production (Greenfield *et al.* 1981).

Indirect methods of assessing insulin sensitivity tend to have the advantage of being less resource intensive but are typically less accurate and reproducible than glucose clamp studies. In the fasting condition, glucose and insulin concentrations are in a basal steady state where insulin levels are relatively steady and hepatic glucose production is constant. Mathematical transformations, which assume equivalence of peripheral and hepatic insulin resistance, have been developed to produce estimates of

insulin sensitivity. One such example is HOMA-IR (Matthews, 1985), which is calculated as follows:

(Fasting glucose (mmol/L) x Fasting insulin (μU/mL)) /22.5

The normalising factor of 22.5 is the product of a fasting insulin of 5 μU/mL and fasting glucose of 4.5 mmol/L; thus a 'normal' individual would have a HOMA-IR of 1. HOMA-IR correlates well with glucose clamp assessment of insulin resistance (r = 0.88) but is limited in individuals with advanced beta cell failure. 'Quantitive insulin sensitivity check index' (QUICKI) is a variant of HOMA-IR which involves logarithmic transformation of fasting glucose and insulin values (Katz *et al.* 2000*)*.

To overcome the limitations of assessments in the fasting state, Matsuda and DeFronzo developed a method which assesses both hepatic insulin sensitivity (fasting state) and peripheral insulin sensitivity (following oral glucose tolerance test). Subjects fast overnight and, following basal blood samples, are given a 75g oral glucose load. Further sampling takes place at 30 minute intervals for 2 hours. 'Insulin Sensitivity Index - Matsuda' is calculated thus:

10,000/ √([fasting glucose (mg/dL) x fasting insulin (uU/mL)] x [mean glucose during OGTT (mg/dL) x mean insulin during OGTT (uU/mL)])

ISI-Matsuda correlates well with both glucose clamp studies (r = 0.74) and HOMA-IR (r = 0.92) (Matsuda, 1999).

Frequently Sampled Intravenous Glucose Tolerance Tests, using minimal modelling techniques, is another dynamic method for indirectly assessing insulin sensitivity. It has the advantage of being less laborious than glucose clamp studies, can provide a range of information on beta cell function and insulin sensitivity and correlates reasonably well with clamp studies, although insulin sensitivity tends to be systematically underestimated (Muniyappa *et al.* 2007).

1.3 Aromatase

Aromatase is the enzyme responsible for conversion of androgens to estrogens (figure 1.1). As noted previously, in both men and post-menopausal women, estrogen receptor activation is largely mediated by local generation of estradiol (in bone, breast, adipose tissue *etc.*). In this context, it has been suggested that circulating estrogen levels are simply a passive indicator of adipose hormone generation and may not operate as hormones in a classical sense (*i.e.* by exerting an effect at a site distant from their generation) (Simpson *et al.* 2005). Estrogen generation by aromatase is just one of many steroid metabolic pathways active in adipose tissue. *In vivo* assessment of adipose steroid metabolism by measuring arterio-venous difference across subcutaneous abdominal fat, demonstrated the release of estradiol and estrone in both men and women. Testosterone was removed, across the gradient, in men but released in women (Boulton *et al.* 1992).

1.3.1 Aromatase and adipose generation of sex steroids

1.3.1.1 Regulation of aromatase

Aromatase is a microsomal member of the cytochrome P450 superfamily of enzymes, encoded by the *CYP19* gene on chromosome 15q21.2. In humans, aromatase is expressed in ovaries, testes, vascular smooth muscle and adipose tissue (Jones *et al.* 2006). Aromatase is regulated by promoters in a tissue dependent manner (Agarwal *et al.* 1997), with promoter I.4 predominant in adipose tissue where it is activated by class I cytokines (including IL-6, IL-11 and oncostatin M) and TNFα and has an obligatory requirement for glucocorticoids (Zhao *et al.* 1995). Promoter II is regulated by cAMP and gonadotrophins and is predominant in the ovaries; although it is also active, to a lesser extent, in adipose tissue, where PGE2 is believed to drive expression (Simpson *et al.* 2002). In breast cancer, increased aromatase expression in peri-tumoral adipose tissue is marked by a switch from promoter I.4 to promoter II (Zhao *et al.* 1996), raising the intriguing possibility that dysregulated aromatase expression may arise in the pro-inflammatory milieu of obese adipose tissue.

Activation of promoter I.4 requires glucocorticoid, which is borne out by *in vitro* evidence of increased aromatase expression in adipose stromal cells exposed to cortisol (Simpson *et al.* 1981). Inhibition of the cortisol-regenerating 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) activity, by carbenoxolone, was shown to influence the aromatase activity of breast adipose stromal cells *in vitro* (Yang *et al.* 1997). More recent evidence has highlighted depot and gender specificities of cortisol response. *In vitro,* cortisol markedly increases aromatase

expression in subcutaneous preadipocytes in women but not men, whilst in omental preadipocytes, cortisol effects a more modest increase in expression in both men and women (Mcternan *et al.* 2002). Leptin down-regulated 11βHSD1 and aromatase expression in female intra-abdominal preadipocytes but had the opposite effect in men. Estradiol stimulated 11βHSD1 in women but decreased aromatase expression; in men aromatase was up-regulated with no effect upon 11βHSD1. Androgens increased expression of both aromatase and 11βHSD1 in male intra-abdominal preadipocytes (Dieudonne *et al.* 2006).

Obesity has been associated with greater aromatase activity (Kley *et al.* 1980), which may reflect greater adipose mass rather than any up-regulation at a cellular level (Cleland *et al.* 1985). However generalised obesity (as determined by BMI), although not central obesity (as determined by WHR), was correlated with increased subcutaneous adipose expression of aromatase mRNA (Wake *et al.* 2007).

1.3.1.2 Distribution of aromatase in adipose tissue

In adipose tissue, aromatase is principally expressed in preadipocytes and factors which promote adipocyte differentiation, such as PPARγ agonists, reduce its expression (Rubin *et al.* 2000). Aromatase is preferentially expressed in buttock and thigh subcutaneous adipose tissue rather than in the abdominal region (2 – 3 fold difference), with no sexual dimorphism but increasing expression with advancing age (Bulun *et al.* 1994). To assess the prevalent pattern of steroid metabolism, stromal cells isolated from various adipose depots were cultured with tritiated androstenedione to assess the percentage conversion to estrone and 5-alpha-reduced

androgens. Aromatase activity was tenfold greater in the upper thigh and buttock compared to subcutaneous abdominal and omental fat. 5α-reductase activity, similar across all locations, was tenfold greater than aromatase activity in subcutaneous abdominal and omental fat (Killinger *et al.* 1990).

1.3.1.3 Other sex hormone metabolism in adipose tissue

In addition to aromatase mediated conversion of androgens to estrogens, a number of other enzymes, which metabolise sex steroid hormones, are active in adipose tissue. 17-beta hydroxysteroid dehydrogenase type 5 (17β-HSD5, also known as AKR1C3) catalyses the conversion of androstenedione to testosterone; *In vitro* assessment of female preadipocytes has shown greater activity in the subcutaneous compartment, as opposed to the omental depot, in keeping with androgen generation in the subcutaneous fat. The activity of subcutaneous 17β-HSD5 is strongly correlated with BMI and reduced with weight loss (Quinkler *et al.* 2004). This study does not accord with earlier findings which suggested 3-alpha hydroxysteroid dehydrogenase type 3 (3α-HSD3, also known as AKR1C2, which catalyses the inactivation of DHT) is more plentiful than 17β-HSD5 in both the visceral and subcutaneous compartment in women (Blouin *et al.* 2003). Both 3α-HSD3 and 17β-HSD5 were more highly expressed in the subcutaneous adipose. In women with elevated levels of visceral adipose tissue, higher 3α-HSD3 activity was observed in omental preadipocytes (Blouin *et al.* 2003).

17β-HSD3, which also converts androstenedione to testosterone, was found to be expressed in female subcutaneous and abdominal adipocytes at significantly higher

levels than aromatase. This is in keeping with *in vitro* conversion of androstenedione to testosterone occurring at a far greater level than androstenedione to estrone. Intra-abdominal adipose 17β-HSD3 to aromatase mRNA ratio was positively correlated with BMI and WHR, with the converse being true of the corresponding subcutaneous ratio (Corbould *et al.* 2002). In men, primary culture studies have demonstrated greater DHT inactivation (mediated by 3α/β HSD) in mature adipocytes and preferentially in the subcutaneous rather than visceral compartment. Omental 3α/β HSD activity was greater in obese men and positive correlations existed between this activity and androgen metabolite levels (androstene-glucuronide and 3α-diol-glucuronide) in all subjects (Blouin *et al.* 2006). In keeping with this, plasma 3α-diol-glucuronide levels have been positively correlated with visceral adiposity (Tchernof *et al.* 1997) and increase with weight gain (Pritchard *et al.* 1996). Central obesity, but not generalised obesity, is associated with increased expression of 3α-HSD3 and 17β-HSD5, in men and women (Wake *et al.* 2007).

More recently, expression of the estrogen inactivating enzyme estrogen sulfotransferase (EST) has been identified in human subcutaneous adipose tissue, in both men and women (Ahima *et al.* 2011), where it was correlated with TNF-α expression. A murine model of adipose EST over-expression resulted in reduced adipose mass and reduced adipose tissue glucose uptake (Khor *et al.* 2010). The relative importance of adipose EST, in relation to aromatase and other enzymes, remains to be established.

Sex hormone metabolism in adipose tissue is clearly a complex process with several intimately related pathways acting simultaneously to determine the local balance of androgen and estrogen receptor activation.

1.3.2 Aromatase deficiency

Insights into the systemic effects of aromatase action were initially derived from rare examples of human aromatase deficiency and also aromatase knockout mice.

Human aromatase deficiency is extremely rare, with only 7 men and 6 women identified as of 2006 (Jones *et al.* 2006). Men with aromatase deficiency tend to present with central adiposity and body mass index in the overweight range. The characteristic lipid profile is of elevated triglyceride and low HDL, which is generally ameliorated by estrogen replacement. Insulin resistance has been reported in most cases, with evidence of improvement with estrogen replacement (Herrmann *et al.* 2002; Morishima *et al.* 1995), although not all affected men demonstrate insulin resistance (Carani *et al.* 1997).

Aromatase-knockout mice (ArKO) develop exaggerated intra-abdominal adiposity, with increased adipocyte volume and decreased lean body mass. At 1 year, insulin and leptin levels were increased, with evidence of reduced glucose oxidation; these changes were associated with reduced spontaneous physical activity but not with any significant change in dietary intake or resting energy expenditure (Jones *et al.* 2000). More detailed assessment of glucose tolerance, using intraperitoneal glucose tolerance testing, confirmed marked insulin resistance in male ArKO mice (Takeda *et al.* 2003).

Estradiol replacement reversed changes in adiposity and adipocyte size with concomitant reduction in leptin and LPL gonadal adipose mRNA transcript levels. As no effects upon lipolysis or fatty acid oxidation were observed, it seems likely that changes in adipocytes are a consequence of increased uptake of circulating lipid (Misso *et al.* 2003). Male ArKO mice display marked hepatic steatosis accompanied by elevated perilipin 2 and FAS mRNA transcript levels in liver; estradiol replacement reverses both the steatosis and reduces perilipin 2 and FAS transcript levels (Hewitt *et al.* 2004). Taken together, the features of human and murine aromatase deficiency support a central role for estrogens in maintaining metabolic health, although a contribution from changes in circulating androgens cannot be completely excluded.

A number of polymorphisms have been identified in *CYP19*, which are known to influence circulating estradiol levels and, in some cases, bone mineral density. The GG genotype of *CYP19 rs2470152* is associated with 13% higher circulating estradiol levels, and higher lumbar BMD, than the AA genotype in young men (Eriksson *et al.* 2009). Men with high levels of a TTTA repeat polymorphism in intron 4 of *CYP19* (associated with higher aromatase activity in cultured skin fibroblasts), have higher concentrations of estradiol and higher lumbar BMD (Gennari *et al.* 2004), whilst low TTTA repeat quantity is associated with obesity and hyperandrogenaemia in premenopausal women (Baghaei *et al.* 2003). *CYP19 rs2446405* AA genotype is associated with higher insulin sensitivity when compared with the AT genotype in peri-menopausal women. Similarly, the CYP19 rs2414095 AA genotype confers a 2.4-fold increased risk of diabetes compared to those with the AG genotype (Lo *et al.* 2006).

1.3.3 Effect of aromatase inhibitors on steroid hormone concentration

Three 'third generation' aromatase inhibitors are currently in clinical use for the treatment of hormone receptor-positive breast cancer in post-menopausal women: two non-steroidal derivatives, anastrozole and letrozole, and one steroidal derivative, exemestane (figure 1.3). Aromatase inhibitors are associated with lower cancer recurrence rates than the selective estrogen receptor modulator tamoxifen (Dowsett *et al.* 2010) and are therefore considered first-line therapy, where typically a five-year course is recommended. In 2012, 1.14 million community prescriptions were issued for third generation aromatase inhibitors in England (Prescribing and Primary Care Services, Health and Social Care Information Centre, 2013).

Anastrozole appears to be a selective inhibitor of aromatase, with no effect observed upon cortisol (including ACTH-stimulated) and aldosterone concentrations (Buzdar *et al.* 2001). Letrozole, in contrast, was associated with a 26.7% reduction in 8am plasma cortisol (with a similar decline in aldosterone) following 4 months of therapy, in women with metastatic breast cancer (Bisagni *et al.* 1996) and also with significant reductions in ACTH-stimulated cortisol, at 3 months, in women with advanced breast cancer (Bajetta *et al.*1999).

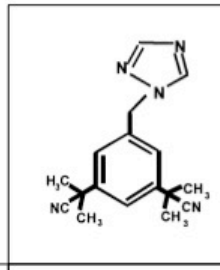
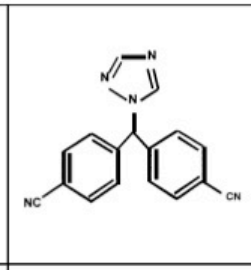
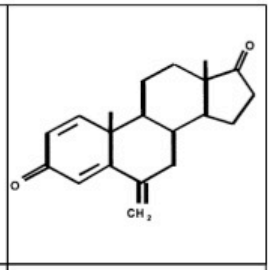
			
Aromatase inhibitor	Anastrozole	Letrozole	Exemestane
Daily dose	1mg	2.5mg	25mg
Time to steady-state (plasma)	7 days	60 days	7 days
Half-life	41 hours	2 – 4 days	27 hours
Time to maximal E2 suppression	3 – 4 days	2 – 3 days	7 days

Figure 1.3 Structure and pharmacokinetics of third generation aromatase inhibitors. Adapted from Buzdar *et al.* 2002.

Assessment of the degree and durability of estradiol suppression with aromatase inhibitors is surprisingly limited given their widespread use but is perhaps explained by the methodological difficulties in accurately measuring estrogens towards the lower end of the physiological range. Within the first few months of therapy in breast cancer, greater than 90% suppression of circulating estradiol is achieved with letrozole and anastrozole. Suppression appears to be less complete in healthy male volunteers and also where employed as a potential therapy for male hypogonadism (table 1.2), presumably as a consequence of secondary elevation of LH and increased substrate androgen levels; negative feedback at the pituitary level is primarily mediated by circulating estradiol (Raven *et al.* 2006). Where a compensatory LH rise was prevented by concurrent goserelin acetate (GnRH agonist) administration, greater estradiol suppression (up to 91.6%) was observed in healthy male volunteers (Finkelstein *et al.* 2013). The method used to measure estradiol may be important, as the degree of suppression was 58% with RIA compared to >89% with GC-MS, in one study, suggesting RIA may also measure cross-reacting estrogen metabolites (Santen *et al.* 2007).

Aromatase inhibitor regimen	n	Plasma E2 % fall	Change in plasma T %	Duration of AI Rx	Tissue E2 % fall	Age (years)	E2 pre (pmol/L)	E2 post (pmol/L)	T pre (nmol/L)	T post (nmol/L)	Reference
Post-menopausal women (breast cancer)											
Letrozole 2.5mg daily	13	95.2%*	NA	4 months	97.6%§	76	19.9	0.65	NA	NA	Geisler 2008
Anastrozole 1mg daily	13	92.8%*	NA	4 months	89.0%§	76	19.9	1.1	NA	NA	Geisler 2008
Letrozole 2.5mg daily	96	39.8%*	+46.2%	12 months	NA	61	34.1	20.6	0.45	0.65	Rossi 2009
Anastrozole 1mg daily	34	90.6%*	+3.1%	12 weeks	NA	63	14.4	1.36	1.28	1.32	Bajetta 2002
Healthy male volunteers											
Letrozole 2.5mg daily	10	56.4%*	+90.2%*	4 weeks	NA	29.9	106	46.2	17.4	33.1	Raven 2006
Letrozole 2.5mg daily	10	62%*	+99%*	4 weeks	NA	76.1	NA	NA	NA	NA	Lapauw 2009
Letrozole 2.5mg daily	9	41%*	+128%*	4 weeks	NA	25.9	NA	NA	NA	NA	Lapauw 2009
Letrozole 2.5mg daily	10	56.6%*	+99.6%*	1 week	NA	34	75.3	32.7	17.2	34.3	Lapauw 2010
Healthy male volunteers (receiving goserelin acetate to suppress LH)											
Anastrozole 1mg daily	41/38	72.2%*	-7.2%	16 weeks	NA	32/34	13.2	3.7	1.5	1.4	Finkelstein 2013
Anastrozole 1mg daily	41/38	84.8%*	+20.9%*	16 weeks	NA	34/33	29.0	4.4	6.6	8.0	Finkelstein 2013
Anastrozole 1mg daily	34/43	84.2%*	+8.9%	16 weeks	NA	32/33	43.7	7.3	11.7	12.7	Finkelstein 2013
Anastrozole 1mg daily	38/42	88.5%*	+1.2%	16 weeks	NA	34/33	66.8	7.7	16.3	16.8	Finkelstein 2013
Anastrozole 1mg daily	44/41	91.6%*	+14.8%	16 weeks	NA	33/34	122.2	10.3	27.9	32.1	Finkelstein 2013
Obese hypogonadal men											
Letrozole 2.5mg once weekly	12	38.2%*	+330.5%*	6 months	NA	48.4	123	76	5.9	19.5	Loves 2008
Testolactone 1g daily	6	27.5%*	+39.0%*	6 weeks	NA	35.5	146.8	106.5	10.1	14.0	Zumoff 2003
Elderly hypogonadal men											
Anastrozole 1mg daily	12	34.6%	+66.3%	12 weeks	NA	67	95.4	62.4	11.9	19.8	Leder 2004
Anastrozole 1mg twice weekly	11	37.1%	+30.4%	12 weeks	NA	67	99.1	62.4	13.8	18.0	Leder 2004
Anastrozole 1mg daily	34	20.0%	+47.7%	12 months	NA	66	55.1	44.1	11.1	16.4	Burnett-Bowie 2009

Table 1.2 Effects of aromatase inhibition upon estradiol and testosterone concentration in plasma and tissue (§ refers to intra-tumoral estradiol concentration). * $p < 0.05$. The study by Finkelstein *et al.* compared matched groups (placebo and anastrozole; n refers, respectively, to numbers in each group and ages are reported separately) receiving varying doses of testosterone, which accounts for 5 separate rows. The study by Lapauw *et al.* 2009 compared two separate groups (older and younger men) hence two separate rows.

1.3.4 Metabolic effects of aromatase inhibitors

Despite the biological plausibility of aromatase inhibition leading to increased insulin resistance, no formal assessment of the effects of this medication class in post-menopausal women (the main group exposed to this treatment) has been published to date. A single small study (n=11) longitudinally assessed body composition in post-menopausal breast cancer patients, treated with 3rd generation aromatase inhibitors, showing a small increase in lean mass, with no significant change in body fat over two years (Van Londen *et al.* 2011).

Female rats treated with letrozole develop a phenotype consistent with PCOS, with inguinal fat accumulation, enlarged adipocytes and insulin resistance, as determined by euglycaemic hyperinsulinaemic clamp (Maliqueo *et al.* 2013), thus lending support to the potential for adverse metabolic consequences.

A number of small studies have sought to assess the metabolic effects of aromatase inhibition in men. In young men, 10 weeks of anastrozole did not influence BMI or fat mass (determined by DEXA) nor did it influence carbohydrate or lipid oxidation (Mauras *et al*. 2000). 12 weeks of anastrozole (1mg daily [n=12] or 1mg twice weekly [n=11]) in elderly men with 'mild hypogonadism' (mean age 67) did not result in any significant difference in HOMA-IR or fasting insulin concentration (Dougherty *et al.* 2005). 28 days of letrozole (2.5mg daily) did not result in any change in fasting glucose or insulin in older men (mean age 76.1 [n=10]) but was associated with reductions of 7% and 37% in fasting glucose and insulin, respectively, in younger men (mean age 25.9 [n=10]) (Lapauw *et al.* 2009); serum leptin was also

reduced following aromatase inhibitor therapy in this cohort. Similarly, 7 days of letrozole (2.5mg daily) in younger men (mean age 34) resulted in significantly improved insulin sensitivity as determined by hyperinsulinaemic euglycaemic clamp, an effect which was negated by concomitant estradiol replacement, although this intervention also resulted in testosterone levels lower than baseline (Lapauw *et al.* 2010); a mixed-meal test, following clamp studies, also demonstrated increased post-prandial plasma GIP concentrations and lower triglyceride concentrations.

The current evidence base is limited by the short duration of interventions, small study populations, employment of suboptimal methodologies for assessing insulin sensitivity and in failing to assess the population most commonly exposed to aromatase inhibition (i.e. post-menopausal women).

1.3.5 Aromatase inhibitors as therapy for androgen deficiency

Obesity and type 2 diabetes mellitus are associated with a high prevalence of male hypogonadism. A large community-based cross-sectional study in the United States (n = 1849) reported rates of subnormal free testosterone in non-diabetic men of 26% (lean), 29% (overweight) and 40% (obese); for men with diabetes the proportions were significantly higher across the three weight categories at 44%, 44% and 50%, respectively (Dhindsa *et al.* 2010). Increased aromatase activity, in an inflamed, expanded adipose pool, has been implicated as the potential mechanism for hypogonadism in this context (figure 1.3). Metformin reduces aromatase expression in cultured breast adipose cells through an effect on cAMP mediated expression (Samarajeewa *et al.* 2011), raising the possibility that a similar effect may exist in the

pro-inflammatory milieu of obese adipose tissue. Total body aromatase activity is known to increase in the context of acute illness, lending support to the concept that a pro-inflammatory state is conducive to increased aromatisation of androgens (Spratt *et al.* 2006). Aromatase inhibition has been investigated as a potential therapy for obesity related hypogonadism, with once weekly letrozole (2.5mg) resulting in either normal or supra-physiological free testosterone concentrations in 12 severely obese men over a 6-month period, without a precipitous decline in estradiol (Loves *et al.* 2008). Aromatase inhibitors effectively increase testosterone levels, even in eugonadal men, but the resultant decline in estradiol has the potential to adversely affect bone mineral density, as was the case in a study of 34 hypogonadal older men, treated for 1 year with anastrozole (1mg daily) (Burnett-Bowie *et al.* 2009).

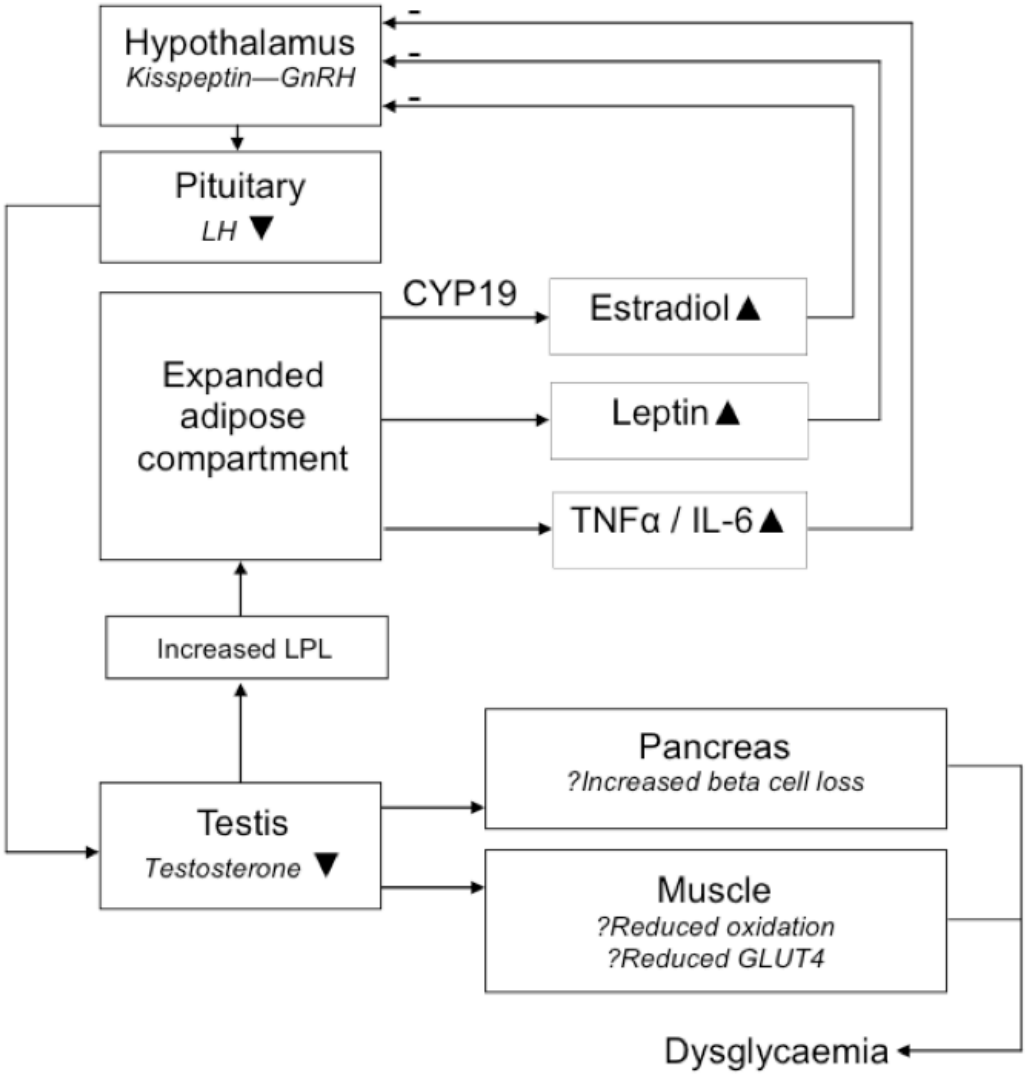


Figure 1.3 Central role for aromatase in the pathogenesis of obesity/diabetes related hypogonadism

1.4 Summary

A substantial body of evidence exists to support a central role for sex steroid hormones in the maintenance of metabolic health and body composition. Perturbations in either circulating levels of sex steroid hormones, or sex hormone action, are associated with adverse changes in body composition, insulin resistance and diabetes mellitus, with some effects likely to be gender specific. Aromatase is a central regulator of circulating androgen and estrogen concentration in men and post-menopausal women and, perhaps more importantly, of tissue concentrations of these hormones. Disruption of aromatase action, be it through genetic mutation or pharmacological inhibition, has metabolic consequences, although as with all manipulations of sex steroid hormones, the relative importance of estrogen and androgen effects can be difficult to discern. This is additionally complicated by the relative balance of androgen receptor, estrogen receptor alpha and estrogen receptor beta activation in any given tissue.

Aromatase inhibitors are a commonly prescribed drug class, making a thorough assessment of their metabolic sequelae desirable. This thesis addresses the influence of sex steroid hormones upon metabolic health, in both men and women, primarily by investigating the effects of pharmacological aromatase inhibition.

1.5 Global hypothesis and aims

This thesis sought to examine the effect of sex steroid hormones upon metabolic health and, in particular, the effects of pharmacological aromatase inhibition.

I hypothesised that:

1. Lower levels of circulating estrogens are associated with poorer metabolic health in both men and women.
2. Aromatase inhibition is associated with deleterious effects upon body composition.
3. Aromatase inhibition is associated with increased insulin resistance.
4. Aromatase inhibition results in alterations in circulating adipokines and cytokines, through an effect on transcription in adipose tissue.

Chapter 2

Materials and methods

2.1 Patient Recruitment

2.1.1 Ethical approvals

Ethical approval for human studies was obtained from the Lothian Research Ethics Committee. The following approvals relate to this thesis:

1. Metabolic effects of aromatase inhibition (07/S1101/32), Chief Investigator: Dr Fraser Gibb
2. Assessing the effects of aromatase inhibition on body fat distribution and insulin sensitivity in post-menopausal breast cancer patients (08/S1101/54), Chief Investigator: Dr Fraser Gibb

2.1.2 Recruitment process

Metabolic effects of aromatase inhibition in men

Healthy male volunteers were recruited through advertisements, placed in local newspapers, and through posters displayed across NHS Lothian and University of Edinburgh sites.

Assessing the effects of aromatase inhibition on body fat distribution and insulin sensitivity in post-menopausal breast cancer patients

Breast cancer patients on aromatase inhibitor therapy were identified by weekly review of case notes at the Breast Cancer Clinic, Western General Hospital, Edinburgh. Age-matched volunteers were recruited from the South East Scotland Breast Cancer Screening Programme through posters displayed in the Breast Screening Centre, Ardmillan House, Edinburgh.

2.2 Tissue Collection

2.2.1. Blood sampling

Whole blood was collected following cannulation of a peripheral vein and stored on ice prior to processing, which occurred within 30 minutes of sample collection. Sarstedt Monovette® blood collection tubes were used. Plasma or serum samples were prepared by centrifugation (12,000 rpm, 10 minutes, 4°C) and stored at -80°C until analysis.

Blood collected for serum was collected in containers containing a separation polyacrylic ester gel. Glucose samples were collected into tubes containing fluoride (1mg/mL blood) and EDTA.

2.2.2 Subcutaneous adipose tissue biopsy

Subcutaneous adipose tissue biopsies were performed in the 'Assessing the effects of aromatase inhibition on body fat distribution and insulin sensitivity in post-

menopausal breast cancer patients' (Chapter 4) and 'Metabolic effects of aromatase inhibition in men' (Chapter 5) studies. Subjects were placed in a recumbent position; the abdomen was exposed and, an area approximately 10 cm lateral to the umbilicus was cleaned with sterile solution (chlorhexidine). Lidocaine 2% (Hameln Pharmaceuticals, Gloucester, UK) was infiltrated intradermally with a 25G needle and into the subcutaneous compartment with a 21G needle (total volume approximately 5mL). Following an interval of 2 minutes (to allow the anaesthetic to take effect), a 12G needle was inserted laterally into the subcutaneous adipose compartment and adipose tissue aspirated into a sterile 50mL syringe, under suction. DEPC water was then drawn into the syringe and the contents expelled over a sterile stainless steel mesh. Visible blood clots were removed with sterile tweezers and the remaining tissue was promptly transferred into a sterile 2 mL eppendorf which was immediately placed on dry ice. Up to three aspirations were performed in order to obtain sufficient tissue. All samples were then stored at -80°C until analysis.

2.3 Materials

2.3.1 Blood collection

- S-Monovette® blood collection system	Sarstedt Ltd., Leicester, UK	

2.3.2 RNA extraction & cDNA synthesis

- RNeasy Lipid minikit	Qiagen, Crawley, UK	Cat. No. 74804
- QIAzol Lysis Reagent	Qiagen, Crawley, UK	Cat. No. 79306
- Chloroform (>99%, PCR reagent)	Sigma, Dorset, UK	C7559-5VL
- Ethanol	Sigma, Dorset, UK	E7023-4X4L

2.3.3 Real time quantitative RT-PCR mRNA transcript assays and solutions

Primers and probes listed in section 2.4 (table 2.2)

- PCR grade water	Roche Diagnostics, Mannheim, Germany	Cat. No. 04 707 494 001
- LightCycler® 480 Probe Master	Roche Diagnostics, Mannheim, Germany	Cat. No. 04 707 494 001
- Universal Probe Library Probes	Roche Diagnostics, Mannheim, Germany	
- Custom primers	Invitrogen Ltd., Paisley, UK	

2.3.4 ELISA

- Estrone ELISA	DRG Diagnostics, Germany	EIA-4147
- Estradiol sensitive ELISA	DRG Diagnostics, Germany	EIA-4399
- Testosterone ELISA	DRG Diagnostics, Germany	EIA-1559
- Androstenedione ELISA	DRG Diagnostics, Germany	EIA-3265
- SHBG Immulite 2000 ELISA	Siemens Healthcare, Llanberis, UK	
- Insulin ELISA	DRG Diagnostics, Germany	EIA-2337
- Insulin ELISA	Abbott Laboratories, Wiesbaden, Germany	08K41-26

2.3.5 Tracer and hyperinsulinaemic euglycaemic clamp

- Accu-Chek Blood Glucose Monitor	Roche Diagnostics Ltd., Burgess Hill, UK	
- 6,6-d_2-glucose	Cambridge Isotopes, CK Gas Products, Hampshire, UK	DLM-349-SP-40
- 1,1,2,3,3-d_5-glycerol	Cambridge Isotopes, CK Gas Products, Hampshire, UK	DLM-1229-SP-10
- Actrapid® Insulin	Novo Nordisk, Crawley, UK	
- Glucose 20%	Baxter, Berkshire, UK	

2.3.6 Gas Chromatography – Mass Spectrometry

- HPLC grade water	Fisher Scientific, Loughborough, UK
- HPLC grade acetonitrile	VWR, Lutterworth, UK
- Heptane	Rathburn, Walkerburn, UK
- Pyridine	Sigma-Aldrich, Dorset, UK
- Acetic anhydride	Sigma-Aldrich, Dorset, UK
- Glucose	Sigma-Aldrich, Dorset, UK
- Glycerol	Sigma-Aldrich, Dorset, UK
- Methane, research grade	BOC, Edinburgh, UK

2.3.7 Liquid Chromatography Tandem Mass Spectrometry

HPLC grade water	Fisher Scientific, Loughborough, UK
HPLC grade methanol	Fisher Scientific, Loughborough, UK
HPLC grade acetone	Fisher Scientific, Loughborough, UK
HPLC grade acetonitrile	Fisher Scientific, Loughborough, UK
HPLC grade hexane	Fisher Scientific, Loughborough, UK
Estrone	Sigma-Aldrich, Dorset, UK
Estradiol	Sigma-Aldrich, Dorset, UK
Testosterone	Sigma-Aldrich, St Louis, USA
Androstenedione	Sigma-Aldrich, Dorset, UK
Formic acid ≥98%	Sigma-Aldrich, Dorset, UK
Triethylamine ≥99.5%	Sigma-Aldrich, Dorset, UK
FMP-TS	Sigma-Aldrich, Dorset, UK
3,4-[$^{13}C]_2$-estrone	Cambridge Isotope Laboratories, Andover, USA
3,4-[$^{13}C]_2$-estradiol	Cambridge Isotope Laboratories, Andover, USA
2,3,4-[$^{13}C_3$]-	Sigma-Aldrich, Dorset, UK

androstenedione

2,3,4-[$^{13}C_3$]-testosterone Sigma-Aldrich, Dorset, UK

2.4 mRNA quantitation

2.4.1 Equipment

All kits for RNA extraction and reverse transcription were from Qiagen, West Sussex, UK.

2.4.2 Buffers and Solutions

2.4.2.1 DEPC water

10 drops of diethylpyrocarbonate (DEPC) were added to 1 litre of distilled water. This was mixed and allowed to stand at room temperature for 24 hours prior to autoclaving. Subsequent storage was at room temperature.

2.4.2.2 10x TBE

Tris base (0.89M), boric acid (0.89M) and EDTA (0.5M, 40 mL) were dissolved in distilled water (800 mL). pH was adjusted to 8.0 by the addition of NaOH (1M) and final volume adjusted to 1 L with distilled water. Storage was at room temperature.

2.4.2.3 0.5x TBE

50mL 10x TBE was diluted with distilled water (950 mL) and stored at room temperature.

2.4.3 RNA Extraction from subcutaneous adipose tissue

Approximately 100 mg of adipose tissue was homogenized in Qiazol Lysis Reagent (1 mL, Qiagen), using a tissue lyser (Tissue Lyser, Qiagen, UK) and incubated for 5 minutes at room temperature. Chloroform (200 µL) was added, mixed and incubated at room temperature for 3 minutes, before centrifugation (12,000 g; 15 minutes; 4°C). The supernatant (600 µL) was removed and replaced by an equal volume of ethanol (70%, *v/v*), which was mixed by pipetting. The solution was transferred to a RNeasy spin column and the eluate discarded after centrifugation (10,000 g; 30 seconds; RT). The column was washed with 700 µL Buffer RW1 and centrifuged (10,000 g; 30 seconds; RT) with the eluate discarded. This was followed by a further washing step with 500 µL Buffer RPE with centrifugation (10,000 g; 30 seconds; RT) and disposal of the eluate. 500 µL RPE buffer was added and the column centrifuged (10,000 g; 2 minutes; RT). Subsequently the RNeasy spin column was placed in a fresh 2 mL collecting tube and any residual RPE buffer removed by centrifugation (16,000 g; 1 minute; RT). The RNeasy spin column was then placed in a fresh Eppendorf (1.5 mL), 40 µL RNase-free water added and incubated at room temperature for 10 minutes. RNA was eluted by centrifugation (10,000 g; 1 minute; RT) and the eluate added back to the column for a further 10 minute incubation at room temperature, to

increase the potential yield of RNA. Finally, RNA was re-eluted by centrifugation (10,000 g; 1 minute; RT) and stored at -80°C.

2.4.4 RNA Quantification

RNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher, West Sussex, UK). Absorbance at 260 nm wavelength (A260) was used to determine concentration. Purity was assessed by the ratio of RNA to DNA (A260/A280); this was regarded as acceptable if between 1.9 and 2.1, effectively excluding significant contamination with protein or solvent.

2.4.5 cDNA synthesis

Total RNA (0.2 - 0.5 μg) was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen). RNA (12 μL) was added to gDNA wipeout buffer (2 μL) and incubated (42°C, 2 minutes) to remove any genomic DNA contaminants. Quantiscript RT buffer, RT primer mix and Quantiscript Reverse Transcriptase were added to each sample (Table 2.1). A negative control without reverse transcriptase was prepared to detect any contamination with genomic DNA ('RT negative' in table 2.1). A further negative control with water and no RNA was used to detect any RNA contamination in the reagents ('Water control' in table 2.1). Samples were incubated (42 °C, 15 minutes; then 95 °C, 3 minutes) in a PCR thermal cycler, before cooling to 4 °C and storage at -20 °C.

	1x reaction volume	RT negative	Water control
500ng RNA	Make up to 12 µL with RNase-free water	Make up to 12 µL with RNase-free water	-
gDNA wipeout buffer	2 µL	2 µL	2 µL
Quantiscript RT buffer	4 µL	4 µL	4 µL
RT primer mix	1µL	1µL	1 µL
Quantiscript reverse transcriptase	1µL	-	1 µL
RNase-free water	-	1 µL	12 µL
Total volume	20 µL	20 µL	20 µL

Table 2.1 cDNA synthesis mixes and controls

2.4.6 Real-time Polymerase Chain Reaction (RT-PCR)

mRNA quantification was performed using real-time PCR on a LightCycler® 480 (Roche Diagnostics Ltd., Burgess Hill, UK) using LightCycler® 480 software version 1.5. Intron-spanning primers (Invitrogen Ltd., Paisley, UK) were designed using the Roche Universal Probe Library Assay Design Center. Primers and probes for genes of interest (and the selected housekeeping gene, cyclophylin A) are summarised in table 2.2. Aliquots from all samples being analysed were pooled and a standard curve created by serial dilution with PCR grade water at the following concentrations: 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512. An 8 µL Mastermix was prepared which constituted: Probe master (5 µL), PCR grade water (2.7 µL), forward primer (0.1 µL), reverse primer (0.1 µL) and probe (0.1 µL); this was added to each well. Water and -RT negative controls (as described in section 2.4.5) were analysed on each plate. Diluted cDNA (2 µL) samples were added to each well, plates were sealed with an optical adhesive cover and then centrifuged (4214 g, 2 minutes, RT) before being run on the PCR system. A single gene was quantified for all samples on one plate. All samples and standards were analysed in triplicate.

Samples were denatured by heating (95 °C, 5 minutes), followed by 50 cycles of PCR amplification: denaturation (95 °C, 10 seconds), annealing (60 °C, 30 seconds) and elongation (72 °C, 1 second). Finally, samples were cooled to 40 °C for 30 seconds. Amplification curves were plotted with cycle number on the x-axis and fluorescence intensity on the y-axis. Excitation was at 483 nm and detection at 533 nm. Triplicates were considered acceptable if standard deviation of the crossing point was <0.4 cycles. The standard curve generated from pooled, diluted samples was fitted

with a straight line and considered acceptable if reaction efficiency was between 1.7 and 2.2.

mRNA was quantified and normalised against the expression of the housekeeping gene, cyclophilin A. This housekeeping gene has been successfully employed by other investigators under similar experimental conditions (Wake *et al.* 2007) and was present in similar abundance to the genes of interest. There have been reports of cyclophilin A upregulation in murine vascular smooth muscle exposed to inflammatory stimuli (Nigro *et al.* 2011) and also of a stimulatory effect of hyperglycaemia in *in vitro* experiments, albeit in monocytes (Ramachandran *et al.* 2012). There is no direct evidence to suggest any effect of sex steroid hormones upon cyclophilin A, in adipose tissue or elsewhere.

Gene: Name Gene accession number	Primer sequence Forward, 5' - 3' ; Reverse, 3' - 5'		UPL probe
ACACA: Acetyl-Coenzyme A carboxylase alpha isoform 1 NM_198834.1	Forward	gatgtggatgatgggctaca	73
	Reverse	tgaggccttgatcattactgg	
ADIPOQ: Adiponectin NM_004797.2	Forward	ggtgagaagggtgagaaagga	85
	Reverse	tttcaccgatgtctcccttag	
PLIN2: Adipocyte differentiation-related protein (Perilipin 2) NM_001122.2	Forward	tcagctccattctactgttcacc	72
	Reverse	cctgaattttctgattggcact	
CYP19A1: Aromatase NM_000103.3	Forward	caaacccaatgaatttactcttga	76
	Reverse	accatggcgatgtactttcc	
AKR1C2: Aldo-keto reductase family 1, member C2 NM_001354.4	Forward	ccatacagaaacttcttttccaca	34
	Reverse	aaccaatggcatgtgagagg	
AR: Androgen receptor NM_000044.2	Forward	gccttgctctctagcctcaa	14
	Reverse	gtcgtccacgtgtaagttgc	
ADRA2A: Adrenergic, alpha-2A-, receptor NM_000681.3	Forward	cttagccccagggcactc	27
	Reverse	caccccagacgaggaaac	
CTNNB1: Catenin (cadherin-associated protein), beta 1 NM_001098209.1	Forward	gctttcagttgagctgacca	21
	Reverse	aagtccaagatcagcagtctca	
CETP: Cholesterol ester transfer protein NM_000078.2	Forward	gatgggagacgagttcaagg	45
	Reverse	tggaagatttcctggttggt	
DGAT2: Diacylglycerol O-acyltransferase homolog 2 NM_032564.3	Forward	gaggggtctgggagatgg	55
	Reverse	ttgagccaggtgacagagaa	
ESR1: Estrogen receptor alpha NM_000125.3	Forward	ttactgaccaacctggcaga	24
	Reverse	atcatggagggtcaaatcca	
ESR2: Estrogen receptor beta NM_001040275.1	Forward	tgggtgattgccaagagc	52
	Reverse	gtttgagaggccttttctgc	
FASN: Fatty acid synthase NM_004104.4	Forward	caggcacacacgatggac	11
	Reverse	cggagtgaatctgggttgat	
GPD2: Glycerol-3-phosphate dehydrogenase 2 (mitochondrial) NM_000408.3	Forward	tcgcgcgtgaggatctat	25
	Reverse	agtcctaaaacagttgcaagagc	

Gene: Name Gene accession number	Primer sequence Forward, 5' - 3' ; Reverse, 3' - 5'		UPL probe
LIPE: Hormone sensitive lipase NM_005357.2	Forward	tgctcggaatcacagacact	90
	Reverse	caggtccatgttgtggatga	
IGF1: Insulin-like growth factor 1 NM_000618.3	Forward	tgtggagacaggggctttta	67
	Reverse	atccacgatgcctgtctga	
IGF1R: Insulin-like growth factor 1 receptor NM_000875.3	Forward	aaaaaccttcgcctcatcc	55
	Reverse	tggttgtcgaggacgtagaa	
LEP: Leptin NM_000230.2	Forward	ttgtcaccaggatcaatgaca	25
	Reverse	gtccaaaccggtgactttct	
LPL: Lipoprotein lipase NM_000237.2	Forward	atgtggcccggtttatca	
	Reverse	ctgtatcccaagagatggacatt	
HSD11B1: 11β hydroxysteroid dehydrogenase type 1 NM_005525.2	Forward	caatggaagcattgttgtcg	20
	Reverse	ggcagcaaccattggataag	
UCP2: Uncoupling protein 2 NM_003355.2	Forward	tgaaagccaacctcatgaca	29
	Reverse	gatgacagtggtgcagaagc	
PNPLA2: Adipose triglyceride lipase NM_020376.3	Forward	ctccaccaacatccacgag	89
	Reverse	ccctgcttgcacatctctc	
PPARG: peroxisome proliferator-activated receptor gamma NM_005037.5	Forward	tgacaggaaagacaacagacaaat	7
	Reverse	gggtgatgtgtttgaacttgatt	
PPARGC1A: Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha NM_013261.3	Forward	tgagagggccaagcaaag	13
	Reverse	ataaatcacacggcgctctt	
SREBF1: Sterol regulatory element binding transcription factor 1 NM_001005291.1	Forward	cgctcctccatcaatgaca	77
	Reverse	tgcgcaagacagcagattta	
SREBF2: Sterol regulatory element-binding transcription factor 2 NM_001005291.1	Forward	cgctcctccatcaatgaca	77
	Reverse	tgcgcaagacagcagattta	
AGT: Angiotensinogen NM_000029.3	Forward	tcaacacctacgtccacttcc	7
	Reverse	gctgttgtccacccagaact	
ADRB1: Beta-1 adrenergic receptor NM_000684.2	Forward	gtggaagatgggtgggttag	7
	Reverse	gagccacgatgatcgatttta	
	Reverse	tctttcactttgccaaacacc	

Gene: Name Gene accession number	Primer sequence Forward, 5' - 3' ; Reverse, 3' - 5'		UPL probe
DGAT2: Diacylglycerol O-acyltransferase 2 NM_032564.3	Forward	gaggggtctgggagatgg	55
	Reverse	ttggacctattgagccaggt	
HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase NM_000859.2	Forward	gttcggtggcctctagtgag	65
	Reverse	gcattcgaaaaagtcttgacaac	
HMGCS1: 3-hydroxy-3-methylglutaryl-CoA synthase 1 NM_001098272.1	Forward	tctgtctactgcaaaaagatccat	59
	Reverse	tgaagccaaaatcattcaagg	
STK11: Serine/threonine kinase 11 (LKB1) NM_000455.4	Forward	caacgaagagaagcagaaaatg	9
	Reverse	tttcctgcatgccacaca	
IL6: Interleukin 6 NM_000600.3	Forward	gatgagtacaaaagtcctgatcca	40
	Reverse	ctgcagccactggttctgt	
PPIA: Peptidylprolyl isomerase A (cyclophilin A) NM_021130.3	Forward	atgctggacccaacacaaat	48

Table 2.2 Primers used for genes of interest analysed by real-time polymerase chain reaction with Roche Universal Probe Library (UPL). Cyclophilin A was the housekeeping gene used in all analyses.

2.5 Enzyme-linked Immunosorbent Assay (ELISA)

2.5.1 Principles of ELISA

Commercially available ELISA kits were used in accordance with manufacturers' instructions. Microtitre wells are coated with antibody directed to an antigenic site on the analyte of interest. Endogenous analyte competes with analyte-horseradish peroxidase conjugate for binding to the antibodies during an incubation phase. Subsequently, sample is removed and the plate washed with a supplied wash buffer (commonly phosphate buffered saline with Tween detergent). A detection solution is added which releases a coloured dye in the presence of peroxidase activity (e.g. 3,3',5,5'-tetramethylbenzidine which is converted to blue-coloured 3,3',5,5'-tetramethylbenzidine diimine). Colour change is inversely proportional to the concentration of analyte in the sample. The reaction is terminated by the addition of sulphuric acid, which leads to a yellow colour change. Light absorption at 450 nm is measured by spectrophotometry.

2.5.2 Individual Assays

All assay ranges, limits of detection and coefficents of variation (CV) are reported as provided by the assay manufacturers.

2.5.2.1 Androstenedione ELISA

Androstenedione was measured using solid phase ELISAs (DRG, Marburg, Germany). The assay range was 0 – 10 ng/mL; limit of detection was 0.019 ng/mL; mean intra-assay CV was 9.1%, mean inter-assay CV was 9.6%.

2.5.2.2 Testosterone ELISA

Testosterone was measured using solid phase ELISAs (DRG, Marburg, Germany). The assay range was 0 – 16 ng/mL; limit of detection was 0.083 ng/mL; mean intra-assay CV was 4.16%, mean inter-assay CV was 9.94%.

2.5.2.3 Estradiol ELISA

Estradiol was measured using high-sensitivity solid phase ELISAs (DRG, Marburg, Germany). The assay range was 0 – 200 pg/mL; limit of detection was < 1.399 pg/mL; mean intra-assay CV was 7.87%, mean inter-assay CV was 8.78%.

2.5.2.4 Estrone ELISA

Estrone was measured using solid phase ELISAs (DRG, Marburg, Germany). The assay range was 2.21 – 1000 pg/mL; limit of detection was 2.21 pg/mL; mean intra-assay CV was 6.5%, mean inter-assay CV was 12.8%.

2.5.2.5 Sex hormone-binding globulin (SHBG)

SHBG was measured using a solid phase, two site chemiluminescent immunometric assay (Immulite 2000, Siemens Healthcare, Llanberis, UK). The assay range was 0.02 – 180 nmol/L; limit of detection was 0.02 nmol/L; mean intra-assay CV was 2.5%; mean inter-assay CV was 4.2%.

2.5.2.6 Insulin

Insulin was measured using a chemiluminescent microparticle immunoassay (Architect, Abbott Laboratories, Wiesbaden, Germany) in the study described in chapter 4. The assay range was 1.0 μU/mL to 300 μU/mL; limit of detection was 1.0 μU/mL; mean intra-assay CV was 3.6%; mean inter-assay CV was 4.7%.

In the study described in chapter 5, insulin was measured by an ultrasensitive insulin ELISA kit (DRG, Marburg, Edinburgh). The limit of detection was 0.07 mU/L; mean intra-assay CV was 5.3%; mean inter-assay CV was 2.7%.

2.6 Measurement of adipokines by multiplex immunoassay

2.6.1 Principles of multiplex immunoassay

Adipokines were measured by Milliplex™ (Merck Millipore, Watford, UK), an immunoassay which utilises fluorescent-coded magnetic beads to simultaneously measure multiple analytes. Capture antibody coated beads are incubated with sample,

washed and a biotinylated detection antibody added. After a further washing step, beads are incubated with a reporter streptavidin-phycoerythrin (SA-PE) conjugate. Finally the beads are passed through an array reader which measures the fluorescence of bound SA-PE. Two 'panels' were used: one to measure leptin, MCP-1 and IL-8 and the other measuring adiponectin and resistin.

2.6.2 Instrumentation

Multiplex assays were performed on a MagPix® system with xPONENT® software, version 4.1 (Luminex, Austin, USA). The instrument comprises two lasers; one to excite internal fluorescent dyes, to identify the analyte of interest, and the other to excite the fluorescent reporter molecule. The latter signal was used for quantification of the analyte of interest. Calibration of the instrument was performed prior to each use.

2.6.3 Leptin, MCP-1 and IL-8

Samples were analysed in accordance with the manufacturer's protocol. Supplied standards covered a concentration range of 38 – 600000 pg/mL for leptin, 1.3 – 20000 pg/L for MCP-1 and 0.64 – 1000 pg/mL for IL-8. Limits of detection, intra-assay CV and inter-assay CV (as provided in the manufacturer's literature) were: leptin 19 pg/mL, 5%, 13%; MCP-1 1.2 pg/mL, 2%, 11%; IL-8 0.3 pg/mL, 3%, 14%.

Assay buffer (200 μL) was added to each well. The plate was sealed and incubated for ten minutes (RT) on a plate shaker, after which assay buffer was removed. 25 μL

of standards or controls were added to the appropriate wells in duplicate. Assay buffer (25 µL) was added to background and sample wells. Serum matrix (lyophilised serum matrix reconstituted in 1 mL deionsed water, 25 µL) was added to background, standard and control wells. Samples (25 µL) were added to sample wells in duplicate. Antibody immobilised beads suspension was sonicated (30 seconds) and vortexed (1 minute). 150 µL of each antibody bead vial (one per analyte) was added to a mixing bottle and made up to 3 mL with bed diluent. Prepared magnetic beads (25 µL) were added to each well, the plate was sealed, wrapped in foil and incubated on a plate shaker for 18 hours at 4°C. Decanting of well contents in subsequent steps was performed with the assay plate on a magnetic plate. The plate was washed three times with wash buffer (diluted 1:10 in deionised water, 200 µL per well). Detection antibodies (50 µL) were added to each well, the plate sealed and then covered in foil for incubation on a plate shaker (1 hour, RT). Streptavidin-Phycoerythrin (50 µL) was added to each well, the plate sealed, covered in foil and incubated on a plate shaker (30 minutes, RT). Well contents were removed and the plate washed (200 µL, three times). Assays were reconstituted in Drive Fluid (100 µL) prior to analysis. A standard curve (logistic 5 point weighted) was constructed and sample concentration calculated by interpolation. Acceptable r^2 for standard curves was >0.99.

2.6.4 Adiponectin (total) and resistin

The method described in section 2.6.3 was employed for measurement of adiponectin and resistin with two exceptions: samples were added at 1:4000 or 1:8000 dilution and no serum matrix was added to background, standard or control wells. Supplied standards covered a concentration range of 0.01 – 160 µg/mL for adiponectin and

2.56 – 40000 ng/mL for resistin. Limits of detection, intra-assay CV and inter-assay CV (as reported by the manufacturer) were: adiponectin 145.4 pg/mL, 5.6%, 15%; resistin 6.7 pg/mL, 6%, 13%.

2.7 Gas Chromatography Mass Spectrometry (GCMS): Quantification of glucose, glycerol, d2-glucose and d5-glycerol

Gas chromatography Mass Spectrometry (GCMS) is employed to separate and quantify chemical species. Gas chromatographic columns comprise a stationary phase (immobile liquid adhering to the column wall) and a mobile phase (the gas passing through the column). When a mixture of chemicals is introduced on to the column, the differing affinity for the mobile and stationary phases determines the rapidity with which individual species reach a detector at the column's end; this is designated as the retention time. Gas Chromatography is allied with Mass Spectrometry, for detection and for specificity, because retention time alone is not sufficiently discriminatory to confidently identify individual chemical species. Upon leaving the GC column, molecules are ionized (either by chemical ionization or electron impact) and these molecular ions (and fragment ions) produce a mass spectrum; essentially a representation of abundance by the mass:charge ratio (*m/z*) for each ion. Prior to analysis, samples are often derivatised to encourage ionization or to stabilise the analyte of interest. In this case, for the quantification of glucose and glycerol, acetate derivatives were formed. Stable isotope tracer studies rely upon slight differences in mass between isotope species, with mass spectrometry the only suitable method of differentiation.

2.7.1 Reagents and standards

- HPLC grade water - Fisher Scientific (Loughborough, UK).
- HPLC grade acetonitrile – VWR (Lutterworth, Leicestershire, UK)
- Heptane – Rathburn (Walkerburn, UK)
- Pyridine, acetic anhydride, glucose, glycerol, $^{13}C_6$-glucose and butanetriol – Sigma Aldrich (Dorset, UK)
- d2-Glucose and d5-glycerol – Cambridge Isotope Laboratories, Inc. (Andover, USA)

Solutions for standard curves and enrichment curves were prepared in HPLC grade water and stored at 4°C, for not more than 2 weeks prior to use.

Standard solutions were:

a. Glucose (5 mg/mL), glycerol (0.025 mg/mL), d2-glucose (0.1 mg/mL) and d5-glycerol (0.025 gm/mL).
b. Internal standard mix – $^{13}C_6$-glucose (5 mg/mL) and butanetriol (0.25 mg/mL).

Reagents (prepared on the day of assay):

a. Acetic anhydride (5%) in heptane.
b. Pyridine:acetic anhydride (1:1, *v/v*).

Standard curves were prepared across the expected plasma concentration ranges for glucose (0 – 9.25 mmol/L), glycerol (0 – 90.5 μmol/L), d2-glucose (0 – 0.018 mmol/L) and d5-glycerol (0.90.5 mol/L). An enrichment curve was prepared representing 0 – 12% enrichment for both glucose and glycerol, with d2-glucose and d5-glycerol, respectively.

Acetonitrile (1 mL) was added to extraction wells (96-well Strata Impact™ protein precipitation plate, Phenomenex, Macclesfield, UK), followed by standards, enrichment standards and samples (200 μL). Internal standard solution (25 μL) was added to the standards and samples but not to enrichment samples. Following a 20-minute incubation period (RT), vacuum was applied (0.3 bar) and extracts collected. Eluates were reduced to dryness under oxygen-free nitrogen (OFN; 37°C). Pyridine:acetic anhydride (200 μL, 1:1 *v/v*) was added to the dry residue and left to stand (RT, 15 mins), forming acetate derivatives (Figures 2.1 and 2.2). The derivatised samples were dried under OFN (37 °C), reconstituted in 5% acetic anhydride in heptane (100 μL) and transferred to GCMS vials (200 μL conical glass inserts, National Scientific, USA). 1 μL of sample was injected onto the GC-MS system.

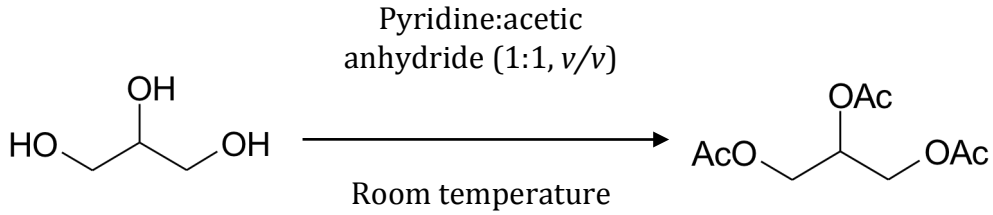


Figure 2.1 Derivatisation of glycerol (left; MW 92.09) with pyridine:acetic anhydride to form glycerol triacetate (right; MW 218.2)

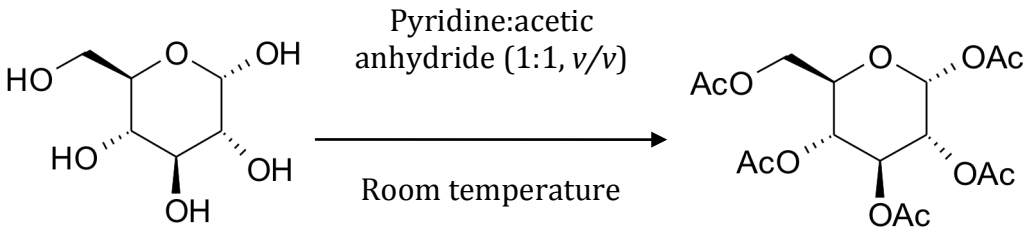


Figure 2.2 Derivatisation of glucose (left; MW 180.16) with pyridine:acetic anhydride to form glucose pentacetate (right; MW 390.34)

2.7.2 Instrumentation

The GC-MS system consisted of a Finnigan GC800^{TOP} GC, an AS800 autosampler and a single quadrupole Voyager mass spectrometer operating with Xcalibur software version 1.2 (Finnigan, now Thermo Scientific, Manchester, UK). The analytical column was an HP-Innowax column (30 m x 0.32 mm x 0.025 μm; Agilent, Berkshire, UK). The sample was injected using splitless injection in a 1 μL volume. Injection temperature was 260 °C. The column oven temperature was 60°C at the point of injection and programmed to reach 150°C at a rate of 30°C per minute; followed by a subsequent rise of 10°C per minute to a target of 260 °C, which was then held for 3 minutes. Interface and source temperatures were 240 °C and 175 °C, respectively. Helium gas was used as the mobile phase at a rate of 2.5 mL per minute.

Mass spectra were acquired using negative chemical ionisation with methane (research grade, BOC, Edinburgh, UK) as the reagent gas at an electron energy of 70 eV. Selective ion monitoring was employed with a total run time of 18 minutes. Glycerol, D5-glycerol and butanetriol derivatives were monitored from 0 – 10 minutes, with glucose, d2-glucose and $^{13}C_6$-glucose derivatives monitored from 10 – 18 minutes. Details of the monitored ions are summarised in table 2.3, glucose derivatives were detected as isomers.

	m/z	Retention time (minutes)
Glycerol triacetate	217	7.15
d5-Glycerol triacetate	222	7.12
Butanetriol triacetate	231	8.16
Glucose pentacetate	287	15.06 & 15.38
d2-glucose pentacetate	289	15.05 & 15.37
$^{13}C_6$-glucose pentacetate	293	15.05 & 15.37

Table 2.3 Monitored ions and retention times

2.8 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) measurement of estrogens

2.8.1 Background

Accurate measurement of estrogens is technically difficult, particularly in men and post-menopausal women in whom circulating levels are often below the limit of quantification of many established assays. LC-MS/MS offers the potential for both sensitive and specific quantification of sex steroid hormones although, as a consequence of inefficient ionisation, derivatisation is often required to introduce a charged moiety. Within our laboratory, a new method was developed using 2-fluoro-1-methylpyridinium-p-toluenesulfonate (FMP-TS), creating estrogen-FMP derivatives which were quantified using triple quadrupole MS in positive electrospray ionisation mode following LC separation (Faqehi *et al.* submitted).

2.8.2 Reagents and standards

- HPLC grade water, methanol, acetone, hexane and water – Fisher Scientific (Loughborough, UK)
- Estrone, Estradiol, 17α-estradiol, formic acid, triethylamine (TEA), FMP-TS – Sigma Aldrich (Dorset, UK)
- 3,4-[^{13}C]$_2$-estrone ($^{13}C_2$E1) and 3,4-[^{13}C]$_2$-estradiol ($^{13}C_2$E2) – Cambridge Isotope Laboratories (Andover, USA).

2.8.3 Extraction

1mL plasma was centrifuged for 20 minutes (8000g, 4°C) and sediment removed. Volume was adjusted to 2mL with water and 200 pg internal standard ($^{13}C2$ labelled estrogen FMP derivatives). Solid-phase extraction was performed under gravity using Oasis MCX cartridges (3 cc/60 mg, Waters, Milford, USA). Cartridges were conditioned with methanol (2 mL) then water (2 mL) before samples were loaded (2 mL). Cartridges were washed with FA (2% v/v, 2 mL) and eluted in methanol (2 mL) before being reduced to dryness under OFN (40 °C) in preparation for derivatisation.

2.8.4 Derivatisation

FMP (50 µL; 5mg/mL in acetonitrile containing TEA [1%]) was prepared immediately prior to reaction and added to the extract. The mixture was vortexed (10 s) and incubated (40°C, 15 minutes) before mobile phase (water: methanol 65:35 containing FA 0.1%, 50 µL) was added to quench the reaction. Figure 2.3 shows the example of an estrone-FMP derivative.

Standard curves were prepared with aliquots containing estrogens at 0, 10, 50, 250, 500, 750 and 1000 pg with internal standard (1000 pg).

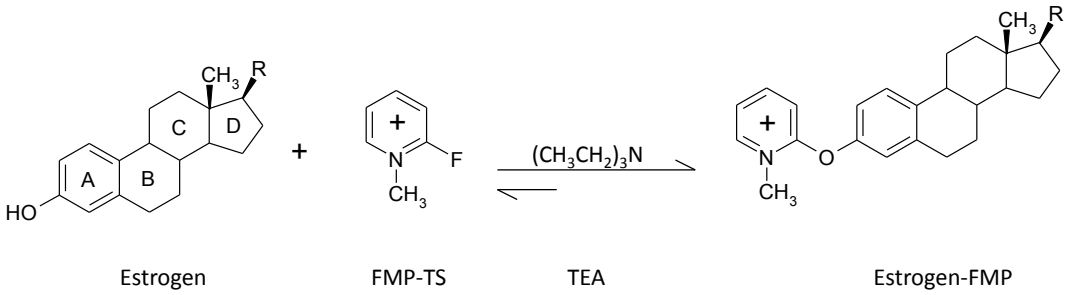


Figure 2.3 Formation of the estrone-FMP derivative following reaction with FMP-TS in the presence of TEA (From Faqehi *et al.*)

2.8.5 Instrumentation

20 µL sample was injected into an Acquity UPLC® BEH C_{18} column (50 x 2.1 mm, 1.7 µm, Waters, Milford, USA) with an isocratic solvent system of water: methanol (65:35), containing FA (0.1%, 0.4 mL/min). The column and autosampler temperatures were 25°C and 10°C, respectively. Estrogen FMP derivatives were quantified using triple quadrupole MS (QTrap 5500, AB Sciex, Warrington, UK) in positive electrospray ionisation mode, using Analyst software version 1.5.1 (AB Sciex, Warrington, UK).

2.8.6 Assay performance

The limit of detection for FMP derivatives of estrone and estradiol was 2 pg/mL; the corresponding limit of quantification was 5 pg/mL At the limit of quantification, intra-assay CV was 12% and 11% for estrone and estradiol, respectively; inter-assay CV was 15% ad 13%, respectively (Faqehi *et al.,* submitted).

2.9 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) measurement of androgens

2.9.1 Reagents and standards

- HPLC grade water, methanol and formic acid– Fisher Scientific (Loughborough, UK)

- Testosterone, androstenedione, 2,3,4-[$^{13}C_3$]-testosterone and 2,3,4-[$^{13}C_3$]-androstenedione – Sigma-Aldrich, Dorset, UK

2.9.2 Extraction

Solid phase extraction was employed using Oasis HLB 30mg cartridges (Waters, Manchester, UK). Cartridges were primed with methanol (1 mL) followed by water (1 mL). 500 µL of sample was enriched with internal standard (1ng) and water added (500 µL). Samples were mixed, loaded onto extraction cartridges and a wash step performed (50% methanol in water, 1 mL); analytes were then eluted with methanol (1 mL). Eluate was dried under OFN (37°C) and then reconstituted in mobile phase (100 µL)

2.9.3 Standard curves

Standard curves were generated for testosterone and androstenedione with the addition of 1ng internal standard across the following concentration ranges:

- Testosterone : 1, 2, 3, 5, 7.5, 10, 12.5, 15 ng/mL
- Androstenedione: 0.1, 0.2, 0.5, 1, 2, 3, 4, 5 ng/mL

2.9.4 Instrumentation

10 µL sample was injected into an Acquity UPLC® (Waters, Manchester, UK) using a Kinetex C_{18} column (150 x 3 mm, 2.6 µm, Phenomenex®, Macclesfield, UK) at

35°C. Elution was achieved with a 9 minute linear gradient from 30:70 to 80:20 (methanol: water with 0.1% FA), with a flow rate of 250 μL/min applied. Initial hold was 1 minute, then the gradient applied for 9 minutes, with a further hold until 16 minutes. Androgens were quantified using triple quadrupole MS (QTrap 5500, AB Sciex, Warrington, UK) in positive APCI mode, using Analyst software version 1.5.1 (AB Sciex, Warrington, UK).

2.9.5 Assay performance

The limits of detection for testosterone and androstenedione were 0.003 ng/mL and 0.08 ng/mL, respectively; the corresponding limits of quantification were 0.005 ng/mL and 0.125 ng/mL, respectively. At 1.8 ng/mL, the intra-assay CV was 2.8% and inter-assay CV 10.0% for testosterone. At 0.18 ng/mL, the intra-assay CV was 5.4% and inter-assay CV 19.3% for androstenedione.

Chapter 3

Associations between circulating sex steroid hormones and cardiometabolic risk in older men: cross-sectional study

3.1 Background

Several studies have sought to investigate relationships between sex steroid hormones and cardio-metabolic risk factors, as well as incident diabetes (Ding *et al* 2006). Frequently the focus has been on androgens in men and estrogens in women, although increasingly the role of both hormone classes has been investigated across genders (Jasuja *et al.* 2013; Oh *et al.* 2002). In addition, lower concentration of sex hormone-binding globulin (SHBG) has been consistently associated with diabetes risk in both men and women (Wallace *et al.* 2013).

SHBG is a 90-KDa glycoprotein which avidly binds testosterone and, with approximately half the affinity, estradiol. Only 2 - 3% of plasma testosterone circulates freely, the remainder is bound with low-affinity to albumin or with high-affinity to SHBG (de Ronde *et al.* 2006). Conventionally regarded as simply a transport protein, increasing evidence suggests SHBG may have an active role in cellular uptake of sex steroids and may be capable of signalling via a G-protein coupled receptor (Rosner *et al* 2010). Whether SHBG simply reflects insulin resistance or has a causative role in diabetes development is unresolved, although recent Mendelian randomisation studies appear to support a protective effect with respect to diabetes development (Ding *et al.* 2009; Perry *et al.* 2010).

Lower total testosterone concentration, a measure of both bound and unbound hormone, has frequently been associated with greater adiposity, reduced lean mass, greater insulin resistance and higher diabetes risk in men (Kelly & Jones 2013). Measuring free testosterone is technically challenging and so it is often calculated indirectly by use of a mathematical

manipulation, which takes account of SHBG and albumin concentrations (Vermeulen *et al.* 1999).

Estradiol levels are typically higher in men with T2DM (Ding *et al.* 2006) and, similarly, higher estrone was associated with an increased risk of developing diabetes in the Framingham cohort, even after adjustment for potential confounders (Jasuja *et al.* 2013).

As detailed in Chapter 1, examining the metabolic effects of androgens and estrogens is complicated by their substrate-product relationship, with estradiol and estrone produced by aromatisation of testosterone and androstendione, respectively. It is often difficult to determine the extent to which effects are a consequence of androgen or estrogen changes. This study sought to examine the relationship between estrogens, androgens and SHBG, as well as their associations with metabolic parameters, in a cohort of 197 elderly men.

3.2 Hypothesis and aims

3.2.1 Hypothesis

The principal hypothesis investigated in the study was:

Lower circulating estrogens, androgens and SHBG are associated with an adverse cardio-metabolic phenotype in older men.

3.2.2 Aims

The aims of the study were to:

1. Determine associations between circulating sex steroid hormones.
2. Assess determinants of estrogen levels in men.
3. Determine associations between sex steroid hormones, SHBG and cardio-metabolic risk factors.

3.3 Methods

3.3.1 Study title and ethical approvals

Study Title: Associations between circulating sex steroid hormones and cardiometabolic risk in older men: cross-sectional study

This cohort was initially established in the early 1990s and included men born between 1920 and 1930 in East Hertfordshire (Hales *et al.* 1991). In 1997, surviving members of this cohort were approached to take part in a further study, which involved administration of 0.25mg dexamethasone at 2300h followed by blood sampling on the following morning (Reynolds *et al.* 2001). This study used stored plasma samples and previously collected data, with ethical approval from the east Hertfordshire ethics committee as granted at the time of the earlier studies.

3.3.2 Study design

3.3.2.1 Subjects: recruitment, inclusion and exclusion criteria

197 subjects were recruited from a cohort established in 1991, whose inclusion criteria were:

- Born in East Hertfordshire between 1920 and 1930
- Birth weight and weight at 1 year recorded

Subjects from this cohort were recruited again in 1997, with the following exclusions:

- Clinical evidence of pituitary or adrenal disease
- Oral glucocorticoid therapy within the past 3 months

3.3.2.2 Study protocol

The following parameters were recorded in 1991:

- Height, weight, waist and hip circumference.
- Seated blood pressure (average of 2 measurements, Dinamap).
- 75g oral glucose tolerance test (men with known diabetes excluded).
- Measurement of glucose (hexokinase method) and insulin (two site immunometric assay) at time points 0, 30 and 120 minutes.
- Fasting plasma triglyceride and cholesterol profile.

The following parameters were recorded in 1997:

- Height, weight, waist and hip circumference.
- Fasting blood samples taken at 0900 following 0.25mg dexamethasone administration the previous evening.

Plasma samples were stored at -80°C until analysis.

3.3.2.3 Assays

Estrone, estradiol, androstendione and testosterone were measured using solid phase ELISAs (DRG, Marburg, Germany) as described in detail in Chapter 2. SHBG was measured using a solid phase, two site chemiluminescent immunometric assay (Immulite 2000, Siemens Healthcare, Llanberis, UK); also described in detail in chapter 2.

3.3.3 Data analysis

3.3.3.1 Calculations

Insulin sensitivity was determined by use of the QUICKI calculation (Katz *et al.* 2000), which is known to correlate closely with hyperinsulinaemic euglycaemic clamp results (Mather *et al.* 2001):

QUICKI = 1 / [log (fasting insulin) + log (fasting glucose)]

Free testosterone was calculated from total testosterone, SHBG and albumin concentrations using the Vermeulen equation (Vermeulen *et al.* 1999).

3.3.3.2 Statistical methods

Normal distribution of data was assessed by the Kolmogorov-Smirnov test and log transformations was performed to achieve normal distributions if required. Associations between continuously distributed variables were assessed by the Pearson correlation coefficient (and partial correlation) using IBM SPSS Statistics for Windows (Version 19.0, Armonk, NY: IBM Corp.) software. Statistical significance was accepted at $p < 0.05$.

3.4 Results

3.4.1 Subject characteristics

Subject characteristics (n=197) and biochemistry are summarised in table 3.1.

	Reference range	Mean ± SEM	Range
Age (years)		70.9 ± 0.22	66.0 – 77.0
Total testosterone (nmol/L)	6.9 – 23.9	18.3 ± 0.6	1.1 – 70.6
Free testosterone (pmol/L)	245 - 785	364 ± 1	104 - 1319
SHBG (nmol/l)	6 – 45	40.29 ± 1.70	8 – 306
Androstenedione (ng/ml)	0.3 – 3.9	6.8 ± 0.5	0.5 – 30.5
Estrone (pmol/L)	156 – 573	566 ± 90	2 - 12102
Estradiol (pmol/L)	36.7 – 132.1	10.3 ± 0.8	1.4 – 62.4
BMI (kg/m^2)		26.91 ± 0.26	17 – 42
Waist circumference (cm)		99.35 ± 0.74	65 – 142
Waist-hip ratio		0.931 ± 0.004	0.77 – 1.06
Fasting plasma glucose (mmol/L)		6.1 ± 0.097	3.4 – 17.3
Systolic blood pressure (mmHg)		161.5 ± 1.5	110 – 232
Diastolic blood pressure (mmHg)		89.3 ± 0.8	59 – 121
QUICKI		0.363 ± 0.003	0.25 – 0.48
Triglycerides (mmol/l)		1.57 ± 0.06	0.5 – 5.3

Table 3.1 Subject characteristics presented as mean ± SEM.

3.4.2 Relationship between sex steroid hormones

Reflecting their substrate: product relationships with respect to aromatase, strong positive correlations existed between testosterone and estradiol and between androstenedione and estrone, even after adjustment for waist circumference (table 3.2). The only positive correlation with SHBG was with estrone, which also persisted following correction for waist circumference (table 3.2).

	Free testosterone	Androstenedione	Estrone	Estradiol	SHBG
Free Testosterone		.235*	.293**	.460*	.053^{NS}
Androstenedione	.203*		.301**	.231*	-.094^{NS}
Estrone	.282**	.286**		.460*	.169*
Estradiol	.473**	.239*	.497**		.149^{NS}
SHBG	.013^{NS}	-.141^{NS}	.151*	.158^{NS}	

Table 3.2 Pearson correlations exploring relationship between sex hormone levels (expressed as r values). Results above the line are uncorrected; those below the line are controlled for waist circumference. * $p<0.05$, **$p<0.01$, NS not significant.

3.4.3 Relationship between sex steroid hormones and metabolic syndrome

71 subjects (35%, cases) fulfilled the NCEP ATPIII diagnostic criteria for metabolic syndrome; the remaining 65% were controls. With reference to controls, the metabolic syndrome cases had significantly lower levels of SHBG (mean difference 12.5nmol/L, $p<0.01$), total testosterone (mean difference 4.5 nmol/L, $P<0.01$) and androstenedione (mean difference 2.2 nmol/L, $p<0.05$). A similar trend was noted with free testosterone, although this did not reach statistical significance, and no differences were observed with estrogen levels.

3.4.4 Relationship between androgens and cardiometabolic risk factors

Results are summarised in table 3.3. Androstenedione, free testosterone and total testosterone were all negatively correlated with indices of adiposity. In addition these hormones were all associated with beneficial effects upon glucose metabolism (either fasting glucose or insulin sensitivity), although these relationships did not persist after correction for waist circumference. The only association which persisted following correction for adiposity was between total testosterone and plasma triglycerides.

3.4.5 Relationship between estrogens and cardiometabolic risk factors

Estrone showed a weak inverse association with insulin resistance and plasma triglycerides; only the latter was independent of waist circumference. Estradiol was inversely related to insulin resistance and glucose, independently of waist circumference (table 3.3), SHBG and testosterone.

3.4.6. SHBG and cardiometabolic risk factors

SHBG was inversely correlated with plasma insulin levels ($r = -.256$, $p<0.001$), waist circumference ($r = -.242$, $p<0.001$) and BMI ($r = -.225$, $p<0.05$). Estradiol was not significantly correlated with SHBG although a weak association existed with estrone ($r = .169$, $p<0.05$). SHBG was more closely correlated with features of metabolic syndrome than any of the sex steroid hormones and the inverse associations with insulin resistance, glucose and plasma triglycerides were independent of waist circumference (table 3.3). The inverse relationship between SHBG and plasma triglycerides (but not glucose or insulin resistance) persisted following correction for plasma insulin levels.

	BMI	WC	glucose	QUICKI	TG	HDL	TC	LDL	SBP
Free testosterone	-.182*	-.177*	-.112	.164*	-.155	.043	.002	.035	-.050
Controlled for WC			-.087	.106	-.101	.031	.021	.051	-.049
Controlled for BMI and SHBG			-.063	.015	-.110	.055	.030	.051	.-.049
Total testosterone	-.229§	-.230§	-.032	.147*	-.260§	.006	-.042	.003	-.063
Controlled for WC			-.006	.068	-.198§	-.014	-.031	.012	-.094
Controlled for BMI and SHBG			-.049	.099	-.204*	.089	-.020	.020	-.095
Bioavailable testosterone	-.144	-.133	-.053	.082	-.126	.078	.041	.062	-.045
Controlled for WC			-.046	.024	-.106	.074	.050	.066	-.032
Controlled for BMI and SHBG			-.046	.021	-.115	.090	.050	.063	-.032
Androstenedione	-.176*	-.209*	-.181*	.080	-.197*	.098	.090	.128	.076
Controlled for WC			-.157	.006	-.132	.063	.085	.103	.093
Controlled for BMI and SHBG			-.170	.047	-.171	.149	.080	.098	.007
Estrone	-.122	-.098	-.124	.154*	-.167*	.071	.091	.093	-.018
Controlled for WC			-.114	.128	-.143*	.063	.098	.116	.022
Controlled for BMI and SHBG			-.020	-.008	.020	-.046	.113	.129	.030
Estradiol	-.019	.041	-.220*	.143	-.065	-.015	.020	.041	-.046
Controlled for WC			-.226§	.177*	-.087	-.012	.034	.075	-.048
Controlled for BMI and SHBG			-.116	.045	-.029	-.045	.025	.051	-.021
SHBG	-.225*	-.242*	-.190*	.283*	-.297§	-.095	-.134	-.171*	-.116
Controlled for WC			-.172*	.209*	-.235§	.077	-.119	-.162*	-.092
Controlled for BMI and SHBG									

Table 3.3 Pearson correlations exploring relationship between sex hormones and components of metabolic syndrome (expressed as r values. * p<0.05, **§** p<0.01,

BMI, body mass index; WC, waist circumference; QUICKI, quantitative insulin sensitivity check index; TG, triglyceride; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; TC, total cholesterol SBP, systolic blood pressure

3.5 Discussion

In this cross-sectional study of elderly men, SHBG consistently demonstrated the strongest relationships with features of the metabolic syndrome. Lower circulating androgens, but not estrogens, were associated with indices of obesity. Lower testosterone levels (free and total) were also correlated with lower insulin sensitivity, which is likely mediated by adiposity. The relationship between lower estradiol and lower insulin sensitivity, in contrast, appeared to be independent of adiposity. Obesity has the potential to affect sex steroid hormone levels, through greater aromatase activity, but adiposity is also likely to be influenced by changes in estrogen and androgen action. Substrate availability (androgen) appeared to be of greater importance than adiposity in determining estrogen concentration.

Although associations of sex hormone levels with cardiometabolic risk factors were mostly negated by controlling for waist circumference, the same did not pertain to SHBG. SHBG is principally, though not exclusively, secreted by the liver under the regulation of a variety of hormones, including insulin and estradiol (Plymate *et al.* 1990). *In vitro*, insulin inhibits SHBG secretion in hepatoblastoma cell lines (Plymate *et al.* 1988) and *in vivo* SHBG is reduced during hyperinsulinaemic euglycaemic clamp studies (Katsuki *et al.* 1996). The converse also holds; SHBG levels rose in men when diazoxide was administered to suppress insulin secretion (Pasquali *et al.* 1995). Similarly, IGF-1 suppresses SHBG production in hepatoblastoma cell lines (Crave *et al.* 1995). In premenopausal but not postmenopausal women, estradiol appears to be a positive determinant of SHBG levels, whilst a negative association with testosterone is ubiquitous (Pasquali *et al*. 1997). In hypogonadal and eugonadal men, testosterone supplementation was shown to effect reductions in SHBG (Plymate *et al.* 1983). Here, no significant correlations between SHBG and either

testosterone or estradiol were found, permitting the inference that these hormones do not occupy a central role in the regulation of SHBG in elderly men. A positive association was observed with estrone, perhaps indicating an association of SHBG with aromatase activity in elderly men.

SHBG binds between 40 – 65% of circulating testosterone and 20 – 40% of circulating estradiol (de Ronde *et al.* 2005); when bound these hormones are protected from conversion to inactive metabolites. The prevailing orthodoxy suggests that bound steroid hormones are prevented from leaving the blood stream and interacting with their cognate intra-cellular receptors. The calculation or measurement of 'free' or 'bioavailable' hormone seeks to address this potential confounder by excluding 'inert' bound steroids. (Mendel 1989). However, this concept has been challenged by the discovery that SHBG interacts with megalin, an endocytic receptor, to induce the active cellular uptake of SHBG bound androgens and estrogens in mice (Hammes *et al.* 2005). A widening role for SHBG is also suggested by the identification of a cell surface SHBG receptor, which generates cAMP when occupied by an SHBG-steroid complex (Kahn *et al.* 2002). Mindful of these potential caveats, determining free hormone levels remains a useful clinical tool.

Evidence from *in vitro* studies had suggested increasing levels of SHBG, by virtue of its greater binding affinity for testosterone, might amplify the bioavailable fraction of estradiol (Burke *et al.* 1972). However this does not appear to apply in young eugonadal men, in whom the hypothalamic-pituitary-gonadal axis adjusts to maintain free testosterone levels (de Ronde *et al.* 2005; Vanbillemont *et al.* 2010). Whether SHBG acts as an 'estrogen amplifier' in older men, with potentially less compensatory reserve, remains an important but

unresolved question. SHBG may simply reflect, rather than contribute to, an adverse cardiometabolic profile. However, recent Mendelian randomisation studies have shown that common polymorphisms, resulting in higher and lower SHBG concentrations, are associated with lower and higher risk of developing T2DM, respectively (Ding *et al.* 2009; Perry *et al.* 2010).

The results of this study are congruent with the strong body of evidence supporting an association between SHBG and glucose intolerance. In two separate cross-sectional studies in middle-aged men (mean ages 57 and 60 years, respectively), SHBG associated with insulin resistance, independent of body fat (Tsai *et al.* 2004), and with hyperinsulinaemia (Muller *et al.* 2005). Similarly in elderly men (age 70 – 89), lower SHBG levels were noted in subjects with metabolic syndrome, pre-diabetic states and T2DM, independent of BMI (Kalme *et al.*2005). Prospective follow up of the Massachusetts aging study demonstrated an increased risk of developing T2DM (Stellato *et al.* 2000) and metabolic syndrome (Kupelian *et al.* 2006) in middle aged men with lower baseline SHBG levels, again independent of body mass. A similar study of middle-aged men in Finland attributed an increased risk of diabetes (OR 4.3) and metabolic syndrome (OR 2.8) to those in the lowest SHBG quartile; these relationships were attenuated but remained significant after adjusting for baseline insulin levels and BMI (Laaksonen *et al.* 2004). Results from the Baltimore study of aging (Rodriguez *et al.* 2007) and MRFIT study (Haffner *et al.* 1996) also attest to the strong relationship between SHBG, metabolic syndrome and diabetes, respectively. Nielsen *et al.* (2007) demonstrated an inverse correlation between subcutaneous adipose mass (as determined by MRI) and SHBG though no independent relationship with visceral adipose mass was noted.

Low SHBG is unequivocally associated with a deleterious plasma lipid profile, namely low HDL cholesterol and high triglyceride levels. In both the PRIME study (Bataille *et al.*2005) and the San Antonio Heart Study (Haffner *et al*. 1993) these associations were independent of BMI and insulin concentrations. Whilst our findings confirm the robust association between SHBG and triglyceride, we did not find a significant relationship between SHBG and HDL cholesterol. This discrepancy may be a consequence of the significantly older age in this cohort.

Significant correlations involving total testosterone may substantially reflect SHBG levels thus complicating their interpretation. Free testosterone was associated with reduced waist circumference and body mass index, as well as having a modest positive correlation with insulin sensitivity in this study. The relationship between free testosterone and insulin sensitivity was not independent of waist circumference, which is consistent with previous studies suggesting that free testosterone is independently associated with reduced visceral adiposity (Nielsen *et al.* 2007). In older men a similar relationship has been noted between free testosterone and subcutaneous adiposity (Abate *et al.* 2005). Inhibition of lipoprotein lipase and triglyceride uptake, evident in obese male recipients of testosterone replacement, may provide a mechanistic underpinning of these observations (Marin *et al.* 1995). Previous cross sectional studies have produced evidence of a positive relationship between free testosterone and glucose tolerance. The San Antonio Heart Study demonstrated an inverse correlation between directly measured free testosterone and glucose and insulin levels in middle-aged men (Haffner *et al.* 1994). This association with free testosterone was independent of BMI although a similar study, employing DEXA and CT assessment,

suggested the positive influence of both free and total testosterone was mediated by changes in total body and abdominal fat (Tsai *et al.* 2004). Abate *et al.* (2002) found no relationship between directly measured bioavailable testosterone and insulin resistance. In prospective studies free testosterone has been independently associated with the development of T2DM in middle aged men (Stellato *et al.* 2000). However, the Massachusetts aging study did not detect a relationship between free testosterone and the development of metabolic syndrome (Kupelian *et al.* 2006), whilst the relationship with free testosterone noted by Laaksonen *et al.* (2004) did not withstand correction for BMI. The findings of a recent meta-analysis (Ding *et al.* 2006) support the link between low testosterone and the development of T2DM.

Our failure to detect a relationship between testosterone and either HDL cholesterol or triglyceride contrasts with the findings of Haffner *et al.* (1993) who report increased HDL cholesterol and lower triglyceride being associated with free testosterone levels independent of WHR and insulin resistance. Gyllenborg *et al.* (2001) surprisingly found the opposite with free androgen levels associated with a deleterious pattern of low HDL cholesterol and high triglyceride.

Relationships between estrogens and metabolic parameters are less extensively studied but a meta-analysis, pooling 12 retrospective studies, suggested higher estradiol levels are associated with the development of T2DM (Ding *et al.* 2006). Nielsen *et al*. (2007) found an independent relationship between estradiol and subcutaneous adipose mass in young men which could be explained by an increase in total aromatase activity. In this study an inverse relationship with estradiol and glucose, independent of waist circumference was observed. This relationship was independent of testosterone (free and total) and so does not appear to

simply reflect elevated androgen substrate. Plausible biological mechanisms exist to explain beneficial effect of estradiol upon glucose metabolism independent of altering body fat distribution: estrogens have been shown to augment the insulin response to glucose (Godsland, 2005), whilst upregulation of muscle GLUT4 expression has been attributed to ERα activation in mice (Barros *et al.* 2006).

The positive association between plasma estradiol and HDL cholesterol reported elsewhere (Gyllenborg *et al.* 2001), was not replicated in this study, although plasma estrone was negatively correlated with triglyceride levels.

Interestingly, the relationship between the substrates for aromatase, testosterone and androstenedione, which are converted to estradiol and estrone respectively, were minimally affected by correction for waist circumference or BMI. In postmenopausal women, peripheral aromatisation (as determined by urinary metabolite analysis) has previously been strongly correlated with adiposity (Longcope *et al.* 1986). Our results raise the possibility that, in elderly men, absolute androgen levels are of greater importance and that any predicted gain in aromatase activity accrued with increasing adiposity, may be negated by reduced substrate availability.

The inability to determine the direction of association is a weakness inherent in cross-sectional studies, although it is tempting to speculate, and is certainly plausible, that many of the relationships reported herein may be bi-directional (*e.g.* sex steroids influence adipose deposition which, in turn, influences sex steroid concentration). Reports of the stability of

SHBG (Lapidus *et al.* 1986) and androgens after prolonged storage at -80°C (Kley *et al.* 1985) assuaged concerns around the interval between sampling and analysis in this study. Ideally, all clinical measurements and assays would have been obtained at a single visit. However, as a consequence of the study design, an interval of approximately six years existed between some measurements (as detailed in section 3.3.2.2). The single low dose of dexamethasone (0.25mg) administered to our subjects could conceivably have resulted in minor suppression of adrenal androgens. In young men, 0.5mg dexamethasone administered twice daily for 4.5 days resulted in an approximate 50% decline in androstenedione but no change in testosterone (Lac *et al*. 1999). A potential strength of this study is the relative homogeneity of the cohort, particularly with respect to age, which is likely to have limited confounding effects present in studies surveying wider ranges, although this does limit the generalizability of the findings. Immunometric sex steroid assays are no longer regarded as the gold standard, having been supplanted by sensitive and specific mass spectrometric assays. All results fell within the expected reference range for this population, with the exception of estrone, for which results were significantly higher than would be plausible in men. Whilst absolute values for estrone are therefore unreliable, subsequent correlation findings (*i.e.* with estradiol and androstenedione) indicate overall trends are sound. Problems with estrone results well outside the physiological range attest to the difficulty in measuring estrogens at low concentration with immunometric assays (Huhtaniemi *et al*. 2012). Any analysis of circulating androgens and, particularly, estrogens is ultimately limited by the potential that local steroid concentrations in target tissue may be of greater physiological significance (Simpson *et al.* 2005).

Summary

This cross-sectional survey of sex hormones and features of metabolic syndrome supports many of the key findings of previous investigators; in particular the clear relationship between SHBG and features of the metabolic syndrome. Our results suggest higher levels of both androgens and estrogens may be associated with metabolic health in elderly men; the former potentially through its effects upon fat distribution and the latter independent of this. The lack of association between SHBG and sex hormone levels suggests their role in regulating SHBG is marginal in elderly men. Finally, we raise the prospect that substrate androgen levels rather than adiposity (and attendant higher whole body activity of aromatase) determine estrogen levels; an unexpected finding which requires confirmation with more rigorous methodology.

Chapter 4

The metabolic effects of aromatase inhibition in post-menopausal women: a case-control study

4.1 Background

Pre-menopausal women have a lower risk of T2DM and cardiovascular disease than age-matched men, a protection associated with a gynoid distribution of body fat. Estrogens appear to play a central role in maintenance of gynoid fat distribution, whilst menopause is associated with a switch to android adiposity (Kuk *et al.* 2005) and with convergence to male levels of diabetes risk (Lemieux *et al.* 1994). However, no prospective studies have confirmed the role of menopausal status, as opposed to ageing, in the development of diabetes (Kim *et al.* 2011). Hormone replacement therapy is associated with a significantly reduced risk of diabetes, with a number needed to treat (NNT) of 30 to prevent one case (Kanaya *et al.* 2003). In contrast, endogenous estradiol levels are positively associated with incident T2DM in post-menopausal women, even after adjustment for adiposity and insulin resistance (Kalyani *et al.* 2009).

Manipulation of estrogen receptor signalling has been a central component in the management of 'hormone-receptor positive' breast carcinoma, in post-menopausal women, for over two decades. Over 1.5 million prescriptions are issued annually for hormonal breast cancer therapies in England (Health and Social Care Information Centre, Government Statistical Service, 2012). Both tamoxifen (an ER partial agonist) and third-generation aromatase inhibitors (e.g. anastrozole, letrozole and exemestane) are associated with increased disease-free survival. However, aromatase inhibitor therapy has not been proven to improve overall survival and is associated with an increased risk of cardiovascular disease (Amir *et al.* 2011), when compared to tamoxifen therapy. The effects of aromatase inhibition upon lipid profile have been

widely investigated (Bundred 2005), with mixed results, and do not necessarily represent a class effect.

Post-menopausal plasma estradiol levels are at least 50% lower than those observed in men. It follows that aromatase inhibition, by lowering circulating estradiol, may result in an exaggerated post-menopausal phenotype. This may be particularly pronounced in adipose tissue as aromatase inhibitors have an even greater suppressive effect upon estradiol in breast adipose tissue, than upon circulating estradiol concentration, in breast cancer patients (Geisler *et al.* 2008). The effects of aromatase inhibition upon insulin sensitivity have not been assessed in post-menopausal women. I sought to address this by assessing body fat distribution and cardio-metabolic parameters in breast cancer patients treated with aromatase inhibition, in comparison to age-matched controls, in a case-control study.

4.2 Hypothesis and Aims

4.2.1 Hypothesis

The principal hypothesis investigated in this study was:

> Inhibition of aromatase results in reduced insulin sensitivity and deleterious changes in body composition.

4.2.2 Aims

The aims of the study were to establish whether women taking aromatase inhibitor in comparison with age-matched controls have:

1. different body composition.
2. reduced insulin sensitivity.
3. changes in adipose tissue mRNA transcript levels.
4. differences in circulating adipokine and pro-inflammatory cytokine levels.
5. altered uric acid excretion.

4.3 Methods

4.3.1 Study Title and Ethical Approvals

Study title: Assessing the effects of aromatase inhibition on body fat distribution and insulin sensitivity in postmenopausal breast cancer patients

Principal Investigator: Dr Fraser W Gibb

Ethical approval was gained from the Lothian Research Ethics Committee in October 2008 (LREC number 08/S1101/54). NHS Lothian granted Research and Development approval in October 2008 (R&D number 2007/W/ON/30). The study was carried out at the Wellcome Trust Clinical Research Facility at the Western General Hospital, Edinburgh.

4.3.2 Study design

Study design was that of a case-control study, comparing breast cancer patients currently receiving aromatase inhibitor therapy with age-matched controls.

4.3.2.1 Subjects: recruitment, inclusion and exclusion criteria

20 post-menopausal women were recruited from the Edinburgh Breast Cancer Clinic. The investigator identified eligible patients by screening of case notes; such patients

were provided with written study information and contact details for the investigator, during their annual clinic appointment. The main inclusion criteria were a diagnosis of ER positive breast carcinoma and current aromatase inhibitor therapy (either anastrozole, letrozole or exemestane) for at least one year. 20 age-matched controls were recruited from the South East Scotland Breast Cancer Screening service, by responding to posters or written study information, available in the waiting area of the screening centre.

Exclusion criteria included: metastatic breast carcinoma, significant medical co-morbidities, hormone replacement therapy, previous diagnosis of diabetes mellitus and recent (within 3 months) therapy with glucocorticoids.

4.3.2.2 Study protocol

Subjects attended the clinical research facility at the Western General Hospital (08:30 am) following an overnight fast. Patients were asked to abstain from alcohol, tobacco and caffeine from the evening prior to attendance. During this attendance, subjects received an oral glucose tolerance test, basic anthropometric measurements, subcutaneous adipose needle biopsy and also submitted a 24-hour urine collection. On a separate day, within 2 months of this visit, patients attended the Western General Hospital for a DEXA scan.

4.3.3 Clinical methods

4.3.3.1 Demographic data

Scottish Index of Multiple Deprivation (SIMD) was ascertained for participants based on their current post code.

4.3.3.2 Basic clinical measurements

Systolic and diastolic blood pressure and pulse rate were measured after sitting for at least 10 minutes using a 705IT automatic blood pressure monitor (OMRON Healthcare, Netherlands).

Weight, height, waist circumference and hip circumference were obtained in all subjects.

4.3.3.3 Oral glucose tolerance test

An antecubital vein was cannulated with a 20G cannula and baseline blood samples collected in serum gel and fluoride tubes (Sarstedt Monovette®, UK). A slow infusion of 0.9% saline was commenced to maintain the patency of the cannula. Blood samples were taken at -30, -15, 0, +30, +60, +90 and +120 minutes; in reference to oral administration of 75g of anhydrous glucose. Blood samples collected in fluoride tubes were sent to the hospital biochemistry laboratory for

measurement of glucose. Serum gel tubes were processed immediately by centrifugation (1912g, 10 minutes, 4°C) and serum stored at -80°C for later analysis.

4.3.3.4 Subcutaneous adipose biopsy

At completion of the oral glucose tolerance test, a subcutaneous adipose tissue needle biopsy was performed (as described in chapter 2.2.2), with the exception of two subjects in the control group who opted out. To summarise, following subcutaneous administration of 5mL local anaesthetic (5ml 2% lidocaine, Hameln Pharmaceuticals, Gloucester, UK), a 19G needle was inserted approximately 10cm lateral to the umbilicus and subcutaneous adipose tissue aspirated by vacuum. The samples were collected in sterile eppendorf tubes, stored immediately in dry ice before transfer to -80°C refrigeration. Adipose tissue was later processed and RNA extracted, as described in detail in chapter 2.4.3. Following reverse transcription (described in chapter 2.4.5), PCR was performed to analyse the mRNA transcript levels of a battery of genes related to adipogenesis, steroid metabolism and adipocytokines (described in chapter 2.4.6).

4.3.3.5 24-hour urine collection

Participants were asked to perform a 24-hour urine collection in the day prior to attending the clinical research facility. The volume of urine was measured and 20mL aliquots of urine stored at -20°C for later analysis of uric acid and creatinine (as described in section 4.3.3.7). 2 subjects in the control group were unable to provide 24-hour urine samples.

4.3.3.6 Dual-energy x-ray absorptiometry

On a separate occasion, within two months of the initial study, subjects attended for a dual-energy x-ray absorptiometry scan (Discovery A, Hologic, Bedford MA, USA) for assessment of body composition. The whole body scanning protocol involves a scan time of 165 seconds and exposure to 0.008 mGy ionising radiation. Estimated bone mineral content, fat mass, lean mass and percentage fat are reported for 5 separate compartments: head, trunk, left arm, right arm, left leg and right leg. The proportion of android fat is defined as: (trunk fat+ arm fat) / total body fat. The proportion of gynoid fat is defined as: leg fat / total body fat (Cao *et al.* 2013). Fat distribution index is the ratio of trunk:leg fat (Kirchengast *et al.* 2004).

4.3.3.7 Laboratory assays

Fasting lipid profile (including total cholesterol, triglyceride, LDL cholesterol and HDL cholesterol), uric acid (serum and urine), creatinine (serum and urine) and glucose were all analysed on the Vitros platform (Ortho-Clinical Diagnostics, High Wycombe, UK), by the biochemistry lab of the Western General Hospital.

Insulin was analysed using a chemiluminescent microparticle immunoassay (Architect 8K41, Abbott Laboratories, Wiesbaden, Germany) as detailed in chapter 2.5.2.6.

Adiponectin, resistin, leptin, MCP-1 and IL-8 were measured by multiplex immunoassay (Merck Millipore, Watford, UK) as detailed in chapter 2.6.1.

4.3.4 Data analysis

4.3.4.1 Fractional excretion of uric acid (FEUA)

Fractional excretion of uric acid was calculated as:

$$\text{FEUA (\%)} = \frac{\text{Urine [uric acid] x Plasma [creatinine]}}{\text{Urine [creatinine] x Plasma [uric acid]}} \times 100$$

4.3.4.2 Insulin sensitivity index – Matsuda

'Insulin sensitivity index – Matsuda' was calculated as:

10,000/ √([fasting glucose (mg/dL) x fasting insulin (uU/mL)] x [mean glucose during OGTT (mg/dL) x mean insulin during OGTT (uU/mL)])

4.3.4.3 Statistical methods

All statistical analyses were carried out using IBM SPSS Statistics for Windows (Version 19.0, Armonk, NY: IBM Corp.) software. Data are presented as mean ± SEM unless otherwise stated. Comparisons between cases and controls were

performed using independent-samples Student's t-tests when data were normally distributed. When data were not normally distributed, as determined by the Kolmogorov-Smirnov test, logarithmic transformation was performed and subsequently compared with Student's t-tests if a normal distribution was obtained or by independent-samples Mann-Whitney U tests if data remained not normally distributed. Assessment of the influence of co-variates was performed using Analysis of Co-variance (ANCOVA). Correlation between normally distributed variables was performed using the Pearson's correlation coefficient and the influence of potential confounders was assessed by partial correlation. Statistical significance was accepted at $p < 0.05$.

At the study design stage, power calculations were performed suggesting a sample size of 40 would provide 80% power in detecting a 50% difference in insulin sensitivity and a 10% difference in waist circumference at a significance level of < 0.05.

4.4 Results

4.4.1 Subject characteristics

Anastrozole was the most prevalent aromatase inhibitor (n=12), with the remaining patients receiving letrozole (n=6) and exemestane (n=2). The mean duration of aromatase inhibitor therapy was 27.4 ± 2.8 months. Cases and controls were well matched with respect to age (61.4 ± 1.4 [range 51 – 72] vs. 59.4 ± 1.0 [range 52 – 67] years, p = 0.259) and BMI (27.1 ± 0.8 vs. 26.6 ± 1.0 kg/m^2, p=0.68). No significant difference was observed in SIMD between cases and controls (4806 ± 355 vs. 5013 ± 419, p=0.71). 5 of the 20 cases had previously received systemic chemotherapy, although this was not associated with any significant differences in any of the parameters measured in this study. Regular medication use was largely limited to levothyroxine replacement and anti-hypertensive agents (Table 4.1).

	Case (n = 20)	Control (n = 20)
Levothyroxine	7	1
Anti-hypertensives	4	1
Bisphosphonate	2	0
Anti-depressants	1	3
HMG-CoA reductase inhibitors	0	1

Table 4.1 Medication use in cases and controls during participation in the study

4.4.2 Blood pressure

No significant differences were noted in systolic blood pressure between cases and controls (139 ± 4 vs. 131 ± 3 mmHg, p=0.140). Diastolic blood pressure was significantly higher in aromatase treated patients (82 ± 2 vs. 75 ± 2 mmHg, p <0.05). 4 aromatase inhibitor treated patients were receiving anti-hypertensive medication versus a single patient in the control group.

4.4.3 Fasting lipid profile

No significant differences were observed in fasting lipid profile between cases and controls (Table 4.2).

	Case (n = 20)	Control (n = 20)	p
Total cholesterol (mmol/L)	6.0 (0.1)	5.7 (0.2)	0.18
HDL Cholesterol (mmol/L)	1.6 (0.1)	1.7 (0.1)	0.37
LDL Cholesterol (mmol/L)	3.8 (0.1)	3.5 (0.2)	0.13
Triglycerides (mmol/L)	1.1 (0.1)	1.0 (0.1)	0.53
Cholesterol:HDLC ratio	3.8 (0.2)	3.5 (0.2)	0.19

Table 4.2 Fasting plasma lipid profile data presented as mean (SEM) and analysed by unpaired Student's t-test.

4.4.4 Body composition

No significant differences were noted in basic measures of body composition, as summarised in table 4.3.

More detailed assessment of body composition was obtained by dual-energy x-ray absorptiometry (DEXA). Lean mass was significantly lower in cases than controls across almost all compartments. Peripheral percentage body fat was significantly greater in aromatase inhibitor treated patients than in controls, although no difference was noted in trunk body fat. Whilst differences in percentage fat were observed, no differences in total fat mass were noted. Fat distribution index (the ratio of trunk to leg fat) did not differ between groups. The full DEXA characteristics of cases and controls are summarised in table 4.4.

	Cases (n=20)	Controls (n=20)	p
Height (cm)	159.8 ± 0.9	165.1 ± 1.7	0.001
Weight (kg)	69.2 ± 2.3	72.5 ± 2.9	0.38
BMI (kg/m^2)	27.1 ± 0.9	26.6 ± 1.0	0.68
Waist circumference (cm)	90.1 ± 2.0	88.4 ± 2.6	0.61
Hip circumference (cm)	102.3 ± 1.6	103.4 ± 1.8	0.68
Waist:Hip ratio	0.88 ± 0.01	0.85 ± 0.01	0.23

Table 4.3 Basic anthropometric measurements presented as mean ± SEM and compared by Student's t-test.

	Case (n=20)	Control (n=18)	p
BMC total (g)	1917.3 ± 56.5	2047.8 ± 81.3	0.189
Total body fat (g)	26722.1 ± 1493.7	25613.9 ± 2010.5	0.657
Total body lean (g)	40284.4 ± 929.9	44726.2 ± 1494.3	0.014
Total body % fat	38.4 ± 1.0	34.6 ± 1.3	0.026
Fat distribution index	1.3 ± 0.1	1.3 ± 0.1	0.584
Proportion android fat	0.6 ± 0.01	0.6 ± 0.02	0.909
Proportion gynoid fat	0.4 ± 0.01	0.4 ± 0.01	0.729

Table 4.4 Detailed DEXA body composition analysis in cases and controls. Data are expressed as mean ± SEM and compared by Student's t-test (normally distributed as determined by Kolmogorov-Smirnov test).

4.4.5 Insulin sensitivity

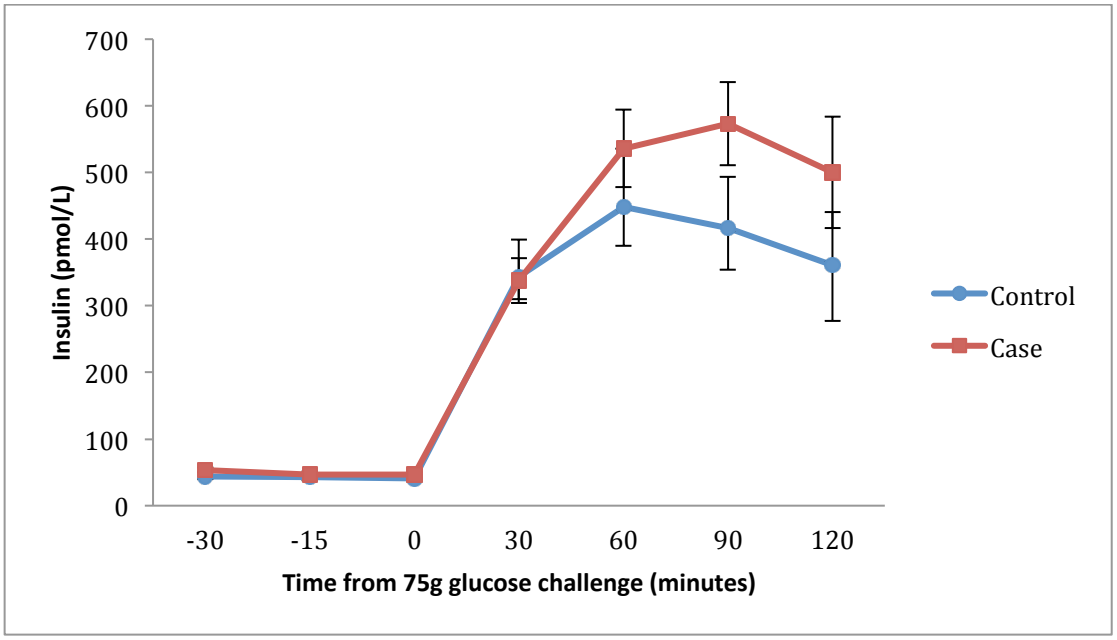
All 40 participants had normal fasting blood glucose levels (*i.e.* < 6mmol/L), with 6 patients in the aromatase inhibitor group displaying impaired glucose tolerance (glucose 7.8 – 11.0 mmol/L at 2 hours following 75g oral glucose) compared to 3 patients in the control group. In addition, 2 patients in the control group had 2-hour glucose levels in the diagnostic range for diabetes (*i.e.* >11 mmol/L).

The pre-specified primary method for determining insulin sensitivity was the 'Matsuda Index' (Matsuda *et al.* 1999). The Matsuda calculation, as originally described, requires paired glucose and insulin measurements at baseline, 30, 60, 90 and 120 minutes following a 75g oral glucose load. Severe haemolysis of plasma samples is recognised to cause significant underestimation of insulin concentration as a consequence of accelerated insulin degradation (Bellomo *et al.* 2012). Baseline insulin concentration was determined from the mean of 3 measures at -30, -15 and 0 minutes, although as a consequence of severe haemolysis, 4 samples in the AI treated group and 1 sample in the control group were not included. During the 2 hours following glucose administration, 7 samples in the AI group and 2 in the control group were excluded due to severe haemolysis. Mean substitution (for the relevant time point) of unusable samples was employed to permit calculation of the Matsuda Index, which did not substantively affect the statistical analysis. Aromatase inhibitor treated individuals were significantly more insulin resistant than controls, based on a 24.3% increase in Matsuda index (Table 4.5), an effect which was independent of age but not body fat percentage. Peak insulin concentration was also significantly greater

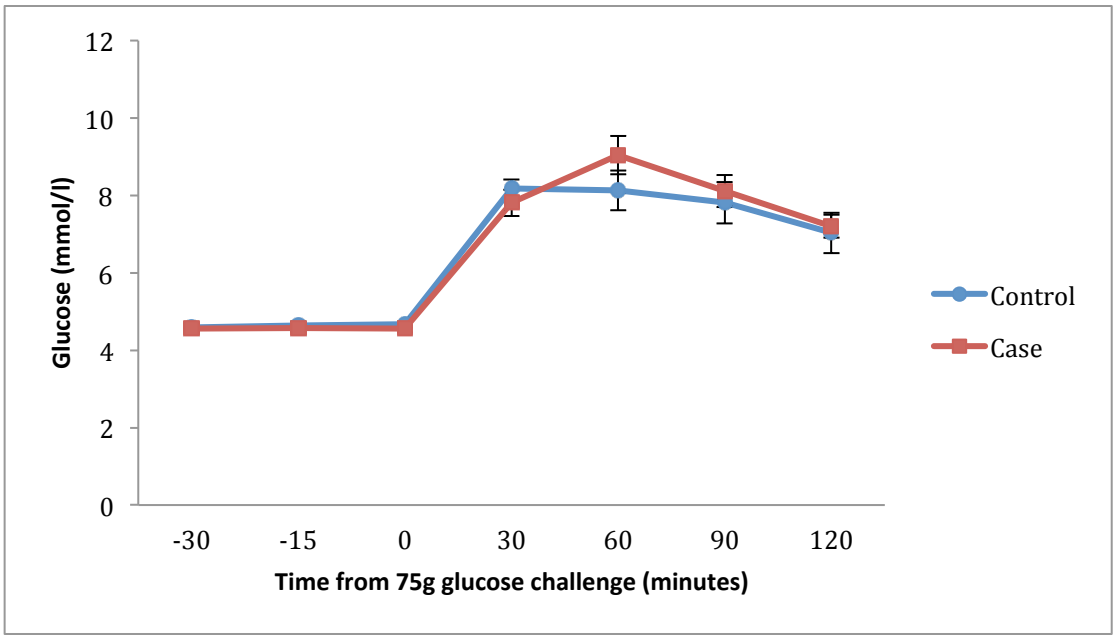
in AI treated patients than in controls, with a similar non-significant trend towards greater insulin AUC (Figure 4.1).

	Case (n=20)	Control (n=20)	P
Matsuda index	5.15 ± 0.45	6.80 ± 0.64	0.041
AUC glucose (mmol/L.120 mins)	913.75 ± 43.07	898.63 ± 37.24	0.792
AUC insulin* (pmol/L.120 mins)	51680 ± 5510	42295 ± 6729	0.079
Fasting glucose (mmol/L)	4.56 ± 0.10	4.66 ± 0.08	0.588
Fasting insulin* (pmol/L)	50.10 ± 4.89	42.55 ± 5.06	0.217
2-hour glucose* (mmol/L)	7.21 ± 0.35	7.04 ± 0.50	0.556
Peak glucose (mmol/L)	9.40 ± 0.39	9.2 ± 0.38	0.744
Peak insulin* (mmol/L)	693.40 ± 78.58	527.60 ± 85.54	0.035

Table 4.5 Indices of glucose tolerance in aromatase inhibitor treated patients and controls presented as mean ± SEM. Compared with independent-samples Student's t-test (data log transformed where not normally distributed as determined by Kolmogorov-Smirnov test*).



A



B

Figure 4.1 Mean plasma insulin (A) and mean plasma glucose (B) across 2-hour 75g OGTT ± SEM. Areas under curves are presented in Table 4.5.

4.4.6 Uric acid

No significant differences were noted in plasma or urine uric acid concentration between cases and control; similarly, no significant difference was observed in fractional excretion of uric acid (Table 4.6). FEUA was negatively correlated with age (Pearson R -0.398, $p < 0.05$) but not with BMI, percentage body fat or insulin sensitivity index. Even after adjustment for age, no significant effect of aromatase inhibition was observed upon FEUA.

	Case (n=20)	Control (n=18)	p
Plasma uric acid (mmol/L)	0.27 ± 0.01	0.26 ± 0.01	0.693
Urine uric acid (mmol/L)*	0.95 ± 0.11	1.1 ± 0.11	0.146
Plasma creatinine (μmol/L)	60.8 ± 1.4	58.9 ± 1.4	0.344
Urine creatinine (mmol/L)	5.2 ± 0.6	4.8 ± 0.6	0.894
FEUA (%)	5.0 ±0.6	4.9 ± 0.5	0.908

Table 4.6 Plasma and urine uric acid levels and calculated fractional excretion of uric acid presented as mean ± SEM. Compared by Independent-samples Student's t-test except where not normally distributed, where Independent-samples Mann-Whitney U Test used*.

4.4.7 Adipokines and pro-inflammatory cytokines

Serum leptin was significantly higher in aromatase inhibitor treated patients (table 4.8), although this relationship did not persist when adjusted for percentage body fat. IL-8, MCP-1, adiponectin and resistin did not differ significantly between cases and controls (Table 4.7). Serum leptin, as expected, was strongly correlated with percentage body fat (Figure 4.2).

	Cases	Controls	p
Leptin (pg/mL)	23485 ± 2826	15527 ± 2286	0.035*
Adiponectin (μg/mL)	43.0 ± 5.9	35.4 ± 3.7	0.516
IL-8 (pg/mL)	7.6 ± 1.1	6.7 ± 0.6	0.871
MCP-1 (pg/mL)	292.5 ± 33.8	268.3 ± 20.2	0.957
Resistin (pg/mL)	22.2 ± 1.1	22.9 ± 1.9	0.828

Table 4.7 Comparison of serum adipokines between aromatase inhibitor treated patients and controls. Data are mean ± SEM. Comparison by independent-samples Student's t-test * or independent-samples Mann-Whitney U Test (where data were not normally distributed even after log transformation).

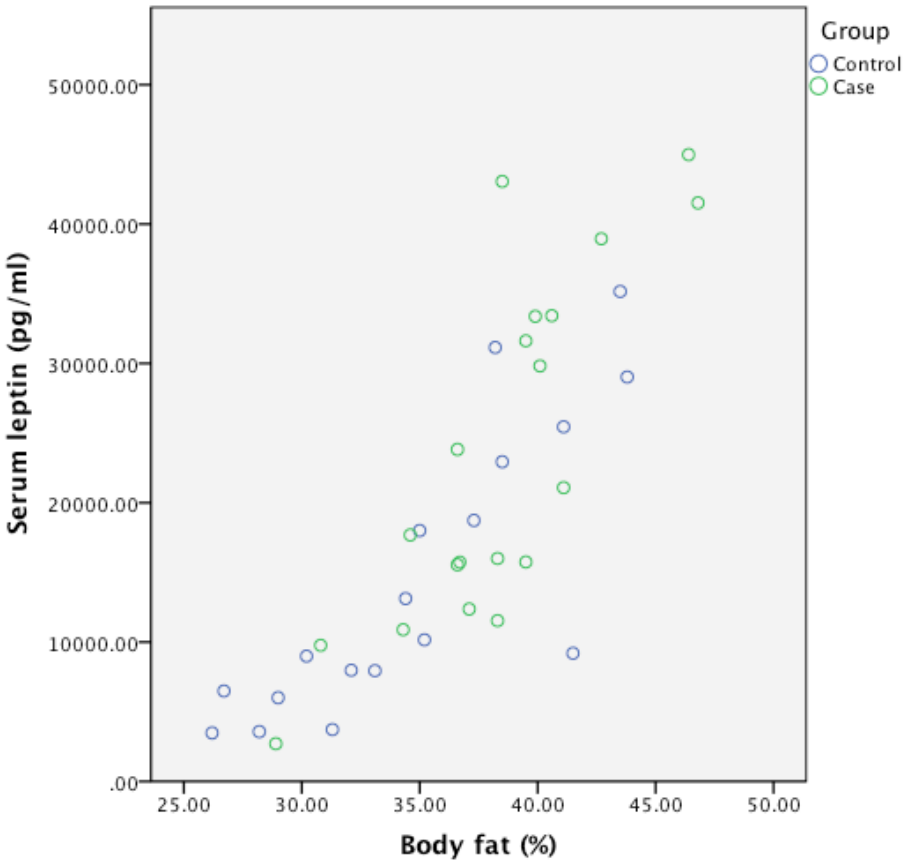


Figure 4.2 The association between percentage body fat and serum leptin levels. Pearson correlation coefficient R 0.826, p <0.001.

4.4.8 Adipose tissue mRNA

mRNA expression was assessed for genes where current evidence suggested a potential regulatory role for sex steroid hormones. Increased subcutaneous adipose tissue expression of LKB1 (32%), β-catenin (27%) and leptin (40%) was observed in aromatase inhibitor treated patients. There were non-significant trends towards greater expression of perilipin 2 (21%), PPARγ (25%) and lipoprotein lipase (14%). The full results for all genes assessed are summarised in table 4.8. A significance level of <0.05 was chosen as all transcripts were selected on the basis of prior evidence of estrogen or androgen responsiveness. Serum leptin was strongly correlated with leptin mRNA expression (Pearson R 0.574, $p < 0.001$) (Figure 4.3). Percentage body fat (Pearson R 0.658, $p < 0.001$) (Figure 4.4) and BMI (Pearson R 0.456, $p < 0.01$) but not WHR (Pearson R 0.320, $p > 0.05$) were significantly correlated with leptin mRNA expression.

	Case (n=20)	Control (n=18)	p
Steroid hormone synthesis and metabolism			
*HSD11B1** 11βHSD1	0.91 ± 0.15	0.78 ± 0.10	0.689
*AKR1C2** Aldo-keto reductase 1C2	1.01 ± 0.20	0.80 ± 0.11	0.845
*CYP19A1** Aromatase	0.66 ± 0.14	0.42 ± 0.09	0.192
Steroid hormone receptors			
AR* Androgen receptor	0.77 ± 0.06	0.71 ± 0.05	0.622
*ESR1** Estrogen Receptor α	0.74 ± 0.10	0.59 ± 0.07	0.176
ESR2 Estrogen Receptor β	0.41 ± 0.03	0.42 ± 0.03	0.919
Adipogenesis, lipogenesis and lipolysis			
ACACA Acetyl CoA carboxylase	0.76 ± 0.09	0.65 ± 0.11	0.443
*UCP2** Uncoupling protein 2	0.44 ± 0.04	0.46 ± 0.03	0.468
FASN Fatty acid synthase	0.65 ± 0.07	0.62 ± 0.07	0.735
LIPE Hormone sensitive lipase	1.28 ± 0.17	0.99 ± 0.14	0.194
PLIN2 Perilipin 2	0.70 ± 0.07	0.55 ± 0.05	0.077
*LKB1** Liver kinase B1	1.44 ± 0.19	0.98 ± 0.15	0.030
CTNNB1 β-catenin	0.62 ± 0.04	0.45 ± 0.02	0.023
*PNPLA2** Adipose triglyceride lipase	0.58 ± 0.07	0.62 ± 0.08	0.724
*PPARG** Peroxisome proliferator-activated receptor γ	0.91 ± 0.11	0.68 ± 0.10	0.087
PPARGC1A PGC-1α	0.67 ± 0.08	0.58 ± 0.08	0.420
Lipid and sterol metabolism			
*CETP** Cholesterol ester transfer protein	0.62 ± 0.14	0.90 ± 0.24	0.343
*LPL** Lipoprotein lipase	1.25 ± 0.07	1.08 ± 0.14	0.086

	Case (n=20)	Control (n=18)	p
SREBF1 Sterol regulatory element binding transcription factor 1	0.86 ± 0.10	0.70 ± 0.12	0.314
*SREBF2** Sterol regulatory element binding transcription factor 2	0.76 ±	0.64 ±	0.303
Adipokines			
*IL6** Interleukin-6	0.87 ± 0.15	1.00 ± 0.20	0.953
*LEP** Leptin	0.92 ± 0.10	0.55 ± 0.06	0.003
ADIPOQ Adiponectin	1.28 ± 0.10	1.17 ± 0.06	0.704§
Miscellaneous			
IGF1R IGF-1 receptor	0.73 ± 0.06	0.62 ± 0.04	0.179
IGF1 Insulin-like growth factor-1	0.67 ± 0.05	0.64 ± 0.05	0.725
AGT Angiotensinogen	0.86 ± 0.08	0.95 ± 0.14	0.591
*ADRA2A** α-2-adrenergic receptor	0.95 ± 0.09	0.83 ± 0.11	0.301
*ARDB1** β-1-adrenergic receptor	0.74 ± 0.07	0.65 ± 0.05	0.380

Table 4.8 Subcutaneous adipose tissue mRNA transcript levels in aromatase inhibitor treated women and controls. Data are presented as means ± SEM (relative to cyclophyllin) and compared with independent-samples Student's t-test (data log transformed where not normally distributed as determined by Kolmogorov-Smirnov test*) or Independent-samples Mann-Whitney U Test where transformation did not result in normally distributed data§.

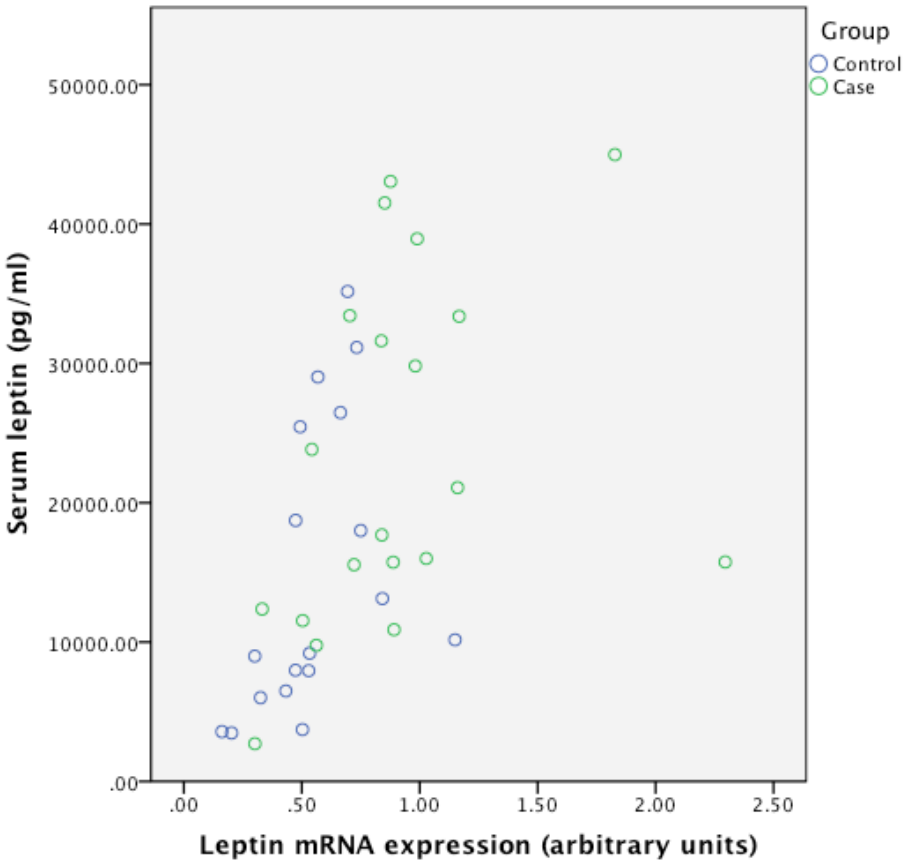


Figure 4.3 The relationship between leptin mRNA expression and serum leptin (R 0.658, p <0.001).

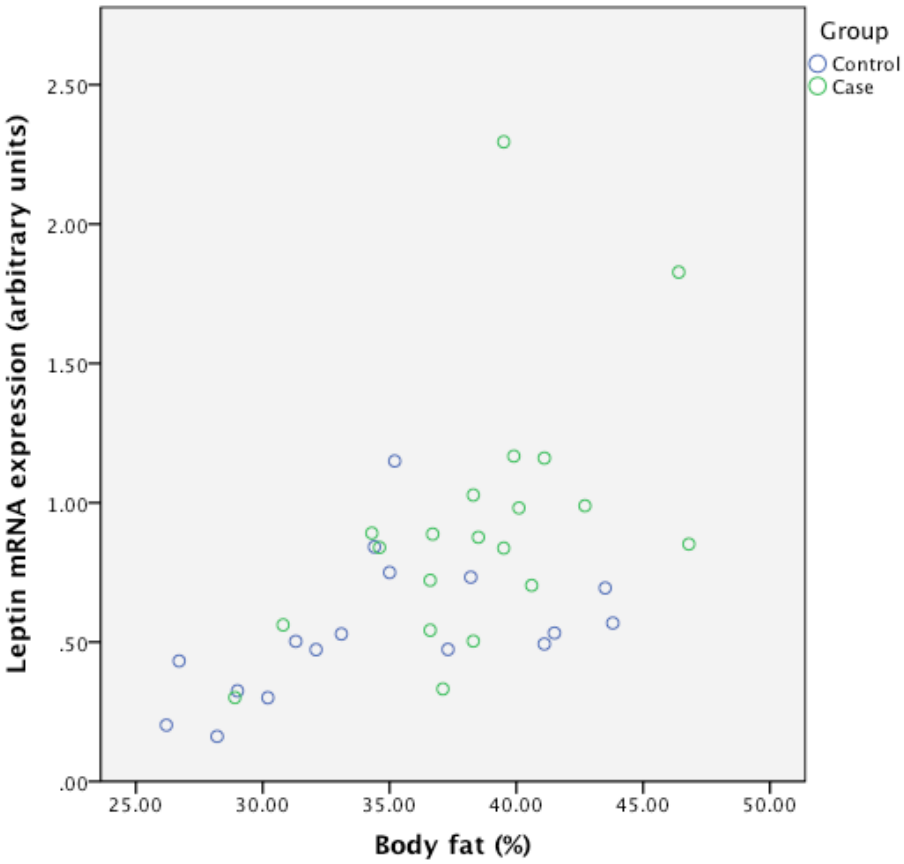


Figure 4.4 The relationship between leptin mRNA expression and body fat percentage (R 0.574, p <0.001).

4.5 Discussion

A substantial body of evidence attests to the importance of androgens and estrogens in modulating body composition and insulin sensitivity. Consequently, it is plausible that aromatase inhibitors, by suppressing estrogens and increasing androgen levels, may exert a deleterious effect upon metabolic health in women. Despite the widespread use of aromatase inhibitors, principally in the treatment of post-menopausal breast cancer, no systematic assessment of their potential metabolic effects has been undertaken. Although aromatase inhibitors are effective in preventing cancer recurrence, this has not been accompanied by improved overall survival, perhaps a consequence of increased cardiovascular events. A meta-analysis comparing outcomes with tamoxifen and aromatase inhibitors identified a 26% increased risk of cardiovascular events in women receiving aromatase inhibitors (Amir *et al.* 2011). Aromatase inhibitors may exert subtle changes in plasma lipid profile, however the evidence for this is inconsistent and does not appear sufficient to account for an excess of cardiovascular disease. This study was designed to assess whether postmenopausal women receiving aromatase inhibitors display significant differences in body composition and insulin sensitivity in comparison to age-matched peers.

Case-control methodology, whilst subject to a number of limitations, was selected on pragmatic grounds. It would have been preferable to perform prospective evaluation of the metabolic effects of aromatase inhibitors by assessing women prior to commencement of therapy and again at an interval following commencement, and to have studied a control group comprising hormone-negative cancer patients. However, this option was considered ethically problematic, as it would have involved inviting

women to take part in a clinic study very shortly after receiving a diagnosis of breast cancer. Furthermore, hormone-negative cancer patients are often offered cytotoxic chemotherapy, which has the potential to influence body composition, thus confounding any comparison with aromatase inhibition. Another potential study design would have been a within-subject comparison performed during therapy and repeated at a pre-specified interval following cessation. This would not have benefitted from a control group comparison and would have failed to detect any residual aromatase inhibitor effects, such as those mediated by changes in body composition, which may persist following treatment cessation.

Patient recruitment achieved satisfactory age matching. Breast cancer patients were recruited from clinics, whilst controls were recruited from the national breast cancer-screening programme. This could potentially have introduced a bias, as attendance at breast cancer screening is positively associated with affluence (Moser *et al.* 2009), with the opposite true of obesity and T2DM (Scottish Diabetes Survey, Monitoring Group, 2008). The Scottish Index of Multiple Deprivation (SIMD) assesses the extent of deprivation, based on 38 measures across 7 categories, in 6505 geographical 'datazones'. No significant difference was noted in SIMD scores between cases and controls, suggesting no major disparity in affluence.

Whilst no specific effort was made to match groups with respect to anthropometric parameters, no significant difference in weight, BMI or waist circumference were observed. Fortunately, this addresses a potentially important criticism of the case-control design, as increasing BMI is associated with a higher risk of breast cancer in post-menopausal women. BMI < 21 kg/m^2 is associated with the lowest risk of

breast-cancer in post-menopausal women, with a non-linear increased risk observed in higher BMI categories (van den Brandt *et al.* 2000). Substantial weight gain following menopause (>10 kg) is also associated with an increased breast cancer risk. Although there is a recognised association between increased height and post-menopausal breast cancer risk (van den Brandt *et al.* 2000), surprisingly the mean height in the control group was significantly higher than breast cancer patients in this study. Whilst height has previously been associated with differences in blood pressure, no association between height and blood pressure was evident in this study.

With the exception of breast cancer, both cases and controls were generally in good health, as mandated by the study exclusion criteria. There was, however, a striking disparity in levothyroxine treated patients between groups (7 cases and 1 control). If levothyroxine therapy is associated with alterations in body composition or insulin sensitivity, this could represent a significant confounder. However, post-menopausal women, treated with thyroidectomy and subsequent levothyroxine, did not develop any significant changes in weight or body composition (as determined by DEXA), over a 12-month period (Kormas *et al.* 1998). A potential association between autoimmune thyroid disease and breast cancer is well recognised, although the direction of the relationship remains unclear. Higher levels of thyroid peroxidase antibody have been observed in patients with malignant breast disease (Giustarini *et al.* 2006) but in a large series of breast cancer patients, primary hypothyroidism was less common than in age-matched controls (Cristofanilli *et al.* 2005).

The decision to include women treated with any of the third generation aromatase inhibitors, rather than electing to assess the effects of a single agent, was expedient in

order to complete the study within a reasonable time; although the majority of cases received anastrozole (n=12). The degree of estradiol suppression appears equivalent between letrozole and anastrozole (Geisler *et al.* 2008), however there is evidence to suggest that letrozole is less specific in its inhibition of steroid production and may exert a suppressive effect upon glucocorticoids and mineralocorticoids (Bisagni *et al.* 1996; Bajetta *et al.* 1996). The sample size was too small to detect any differences between agents, although a more comprehensive assessment of potential differences would certainly be welcome.

Blood pressure displays sexual dimorphism, increases following menopause, varies across the menstrual cycle and falls, in parallel with increased estradiol, in pregnancy; all these factors support a role for estrogens in regulating blood pressure (Dubey 2002). In this study, diastolic (but not systolic) blood pressure was higher in aromatase inhibitor treated women than controls, despite a higher proportion of anti-hypertensive therapy in the former. Interestingly, polymorphisms in *CYP19* have been associated with differences in diastolic blood pressure in women (Peter *et al.* 2005) and identified as potential markers for essential hypertension in both genders (Shimodaira *et al.* 2008). Remarkably, despite concerns regarding increased cardiovascular events, no formal assessment of the effect of aromatase inhibition upon blood pressure has been reported in the literature.

No significant difference in fasting lipid profile was noted between cases and controls, which is broadly in accord with existing evidence. The suggestion that aromatase inhibitors may increase LDL cholesterol is largely based on head-to-head

studies with tamoxifen (ATAC Trialists' Group 2006), however the disparity is most likely a consequence of the lipid-lowering properties of tamoxifen. Prospective evaluation of cholesterol, following anastrozole therapy, failed to detect any significant difference in fasting lipid profile (Van Poznak *et al.* 2012).

Whilst basic anthropometric parameters were no different between cases and controls, more detailed body composition, by DEXA, revealed greater body fat percentage and lower lean mass in aromatase inhibitor treated women. DEXA was selected as the modality for assessment of body composition on the basis of evidence suggesting it accords closely with the reference standard, underwater weighing (Fogelholm *et al.* 1997), as well as providing estimates of body composition across a number of compartments. Only two previous studies have assessed the effect of aromatase inhibition upon body composition and are potentially confounded by patients in the comparator groups receiving tamoxifen. In one study, 11 recently menopausal breast cancer patients receiving aromatase inhibitors were compared with 71 women on alternative therapies (mostly tamoxifen). Over a 24-month period, aromatase inhibitor treated women gained lean mass, whilst non-aromatase inhibitor treated women developed increased fat percentage (van Londen *et al.* 2011). Conversion from tamoxifen to exemestane was associated with a significant reduction in fat mass (and increase in FM/FFM ratio) in 28 post-menopausal breast cancer patients, over a period of 1 year (Francini *et al.* 2006). Prospective evaluation of tamoxifen treated women, over 2 years, failed to show any significant changes in body composition, as assessed by DEXA, albeit with a limited set of parameters reported (Grey *et al.* 1995); however, tamoxifen may be associated with increased visceral adiposity (Nguyen *et al.* 2001). The findings in this study conflict with these earlier investigations,

however elevated serum leptin in aromatase inhibitor treated women corroborates the apparent increase in body fat as determined by DEXA. Furthermore, a trend towards increased LPL mRNA expression in subcutaneous adipose tissue is consistent with the established literature on estrogen effects on adipose tissue (Mauvais-Jarvis *et al.* 2013). ER-α is thought to protect against sarcopenia through activation of Akt and MAPK pathways. HRT has consistently been associated with improvements in lean body mass (Chen *et al.* 2005; Sørensen *et al.* 2001), therefore estrogen suppression, through aromatase inhibition, may result in reduced muscle mass, as suggested from this study. It would be of interest to determine the differential effects of aromatase inhibition upon visceral and subcutaneous adipose volume, by CT or MRI analysis.

Aromatase inhibitor treated women were more insulin resistant than controls, as determined by 'Insulin Sensitivity Index – Matsuda'. This is consistent with the predicted effects of reduced estrogen and increased androgen activity upon skeletal muscle, liver, beta cells and adipose tissue, in women, as described in chapter 1. The largest change appears to have been greater hyperinsulinaemia following a glucose load, suggesting peripheral insulin resistance mediates a large component of the aromatase inhibitor effect. 'ISI – Matsuda' was selected as the primary outcome measure for this study as it integrates information from both the fasting and fed state, to provide an estimate of insulin sensitivity which accords well with gold-standard glucose clamp studies. Clamp studies would have provided more information regarding the relative contribution of hepatic and peripheral insulin sensitivity, as well as effects on lipolysis, however time and cost constraints precluded pursuing this methodology.

Recent observations that uric acid may have a contributory role towards the development of insulin resistance (Johnson *et al.* 2009), paired with a potential role for estrogens in modulating the excretion of uric acid (Yahyaoui *et al.* 2008), raised the possibility that aromatase inhibition may effect suppression of uric acid excretion. In a rat model of hyperuricaemia (induced by high-fructose feeding), lowering of urate with allopurinol was shown to mitigate hypertension, weight-gain, hypertriglyceridaemia and hyperinsulinaemia (Nakagawa *et al.* 2006). Elevated uric acid levels have long been considered a consequence of insulin resistance rather than a cause, however prospective studies have identified uric acid as an independent risk factor for the development of metabolic syndrome in men and women (Sui *et al.* 2008). A study in male to female transsexuals, demonstrated a dose-dependent suppressive effect of estrogen replacement upon serum uric acid, with an accompanying increase in fractional excretion of uric acid (Yahyaoui *et al.* 2008). However, in the current study, no significant difference was noted in either serum levels, or urinary excretion, of uric acid between cases and controls. This suggests that below a threshold estradiol concentration, further suppression does not exert any additional influence upon uric acid.

Serum leptin was significantly higher in aromatase inhibitor treated patients compared to controls, an observation consistent with the increased body fat percentage detected by DEXA. Furthermore, leptin mRNA transcript levels in subcutaneous adipose tissue were also increased in aromatase inhibitor treated women, suggesting an effect in addition to simple expansion of adipose volume. Consistent with this study, aromatase knockout mice, of both sexes, display substantially elevated serum leptin

levels and, in this murine model, central leptin sensitivity appears preserved, on the basis of reduced food intake (Jones *et al.* 2001). Similar findings are observed in aromatase knockout mice with respect to leptin adipose mRNA transcript levels, which are lowered by estradiol administration (Misso *et al.* 2003).

In contrast, the hormonal manipulations undertaken in male-to-female and female-to-male transsexuals, result in increased and decreased serum leptin, respectively (Elbers *et al.* 1997). Omental adipocytes from women, but not men, are stimulated to produce leptin when incubated with estradiol (Casabiell *et al.* 1998). In cultured adipocytes from male rats, DHT exposure reduces leptin mRNA expression, whilst the opposite effect is observed in female adipocytes exposed to estradiol (Machinal *et al.* 1999). A paradox exists between what may be expected from our understanding of the regulation of leptin expression, based on cell culture studies and exogenous administration of sex steroids, and what is observed in aromatase knockout mice and here in aromatase inhibitor treated women.

Aromatase inhibitor therapy, in post-menopausal women, does not appear to exert any influence on adiponectin, as determined by serum concentration and adipose mRNA expression in this study. Serum adiponectin levels are typically higher in women and, in post-menopausal women, transdermal estrogen replacement is associated with an increase in circulating adiponectin (Chu *et al.* 2006). Conversely, in hypogonadal men, testosterone replacement appears to effect a reduction in circulating adiponectin (Lanfranco *et al.* 2004). Given the anticipated changes in adipose and circulating sex hormones, it is perhaps surprising that no influence upon adiponectin was observed. No differences were observed, between groups, in serum IL-8, MCP-1 or resistin

concentrations. We did not assess TNF-α in this study, however TNF-α mRNA expression has been positively correlated with estrogen sulfotransferase (EST) expression in human subcutaneous adipose tissue (Ahima *et al.* 2011). Whether adipose inflammation promotes EST expression or, alternatively, reduced adipose estradiol (through increased EST expression) promotes inflammation, is not clear; it would be of interest to see whether aromatase inhibition influences adipose EST and TNF-α expression.

A range of mRNA transcript levels were assessed in subcutaneous adipose tissue, based on previous evidence of regulation of expression by sex hormones. β-Catenin transcript levels, a central component of Wnt signalling, were higher in AI treated women. β-Catenin has been shown to interact with both ER and AR and appears to exert an effect on transcription factors, which is broadly anti-adipogenic (Singh *et al.* 2006; Kouzmenko *et al.* 2004). LKB1is an upstream activator of AMPK, which promotes fatty acid oxidation and suppresses fatty acid synthesis in adipocytes. Cell culture studies have shown estradiol up-regulates transcription of LKB1 whilst DHT exerts the opposite effect (McInnes *et al.* 2012). Surprisingly, in the context of reduced estrogen and increased androgen levels, AI treated women had significantly higher LKB1 transcript levels compared to controls. To summarise, the statistically significant differences in transcript levels are consistent with an anti-adipogenic effect (increased LKB1 and β -catenin) although leptin mRNA levels were higher. This study has not taken into account the potential effects of EST activity and no direct measurements of adipose tissue sex steroid levels were performed; both of which would contribute useful additional information. Subcutaneous adipose tissue was studied because of the relative ease of acquiring tissue from this compartment, in

contrast to visceral adipose. However, the visceral and subcutaneous adipose compartments have distinct characteristics and ideally assessment of the effects of aromatase inhibition would have been undertaken.

Summary

The findings in relation to decreased lean mass and insulin sensitivity highlight the potential role of sex hormone manipulation upon skeletal muscle, the main source of peripheral glucose uptake. Indeed, in men at least, it has been suggested that skeletal muscle is the largest pool of non-gonadal aromatization (Longcope *et al.* 1978). Further characterisation of the effects of aromatase inhibition upon skeletal muscle in women, through analysis of gene expression and quantification of intramyocellular lipid would be of particular interest. As hypothesised, aromatase inhibition was associated with increased insulin resistance and adverse changes in body composition, although this manifested as reduced lean mass rather than, as predicted, a shift from gynoid to android adiposity. These results should prompt a more comprehensive assessment of the metabolic effects of aromatase inhibitors in their main target population.

Chapter 5

The metabolic effects of short-term aromatase inhibition in healthy male volunteers: a double-blind, placebo-controlled, crossover study

5.1 Background

A substantial body of evidence, at both a cellular and whole organism level, has accumulated in support of the influence of sex steroid hormones upon energy metabolism (Mauvais-Jarvis *et al*. 2013; Kelly & Jones 2013). However the emphasis of investigation is frequently focussed on estrogens in females and androgens in males, with much less information available regarding the role of androgens in women and, even less regarding estrogens in men. In view of the interdependence of substrates and products, particularly of aromatase, most experimental manipulation is complicated, by exerting significant effects upon both androgens and estrogens. Greater insulin resistance, visceral adiposity, diabetes risk and cardiovascular morbidity are observed in men, when compared to age-matched premenopausal women; with lower estrogen levels implicated as a potential explanation for this disparity (Moran *et al.* 2008).

In clinical and experimental medicine, relatively little attention has been paid to the potentially deleterious effects of estrogen deficiency in men. Male aromatase knockout mice display a characteristic phenotype of increased adiposity, hepatic steatosis, reduced lean mass and insulin resistance, which is largely reversible following estradiol replacement; a similar phenotype is observed in rare human cases of aromatase deficiency (Simpson *et al.* 2005). A handful of small studies have investigated the effects of pharmacological aromatase inhibition in men, resulting in either improved insulin sensitivity (Lapauw *et al.* 2009; Lapauw *et al.* 2010) or no significant change in insulin sensitivity (Dougherty *et al.* 2005). An elegant study sought to determine the relative contribution of androgens and estrogen to changes in

body composition, by suppressing LH (with goserelin) and administering varying doses of testosterone, with or without aromatase inhibitor; increasing body fat (particularly intra-abdominal) was associated with estrogen suppression (Finkelstein *et al.* 2013). It is unsurprising that changes in the balance of estrogen and androgen signalling should effect changes in adipose volume, as several genes involved in adipogenesis are known to be regulated by sex steroid hormones (section 1.1).

Little is currently known about the specific effects of aromatase inhibition upon transcriptional regulation in adipose tissue and no detailed assessment of their influence upon glucose and fat metabolism has been undertaken. In the previous chapter, aromatase inhibition was associated with reduced insulin sensitivity in post-menopausal women. The current chapter describes a study which sought to further characterise the metabolic effects of aromatase inhibition, employing superior study design (double-blind, placebo-controlled) and gold standard methodology for assessing insulin sensitivity (hyperinsulinaemic euglycaemic clamp with stable tracers).

5.2 Hypothesis and aims

5.2.1 Hypothesis

The principal hypothesis investigated was:

Aromatase inhibition in healthy men results in increased insulin resistance, associated with reduced estrogen action in subcutaneous adipose tissue.

5.2.2 Aims

The aims of the study in men were to:

1. Determine whether short-term aromatase inhibition adversely effects insulin sensitivity, resulting in altered lipid and glucose homeostasis.
2. Assess the effects of aromatase inhibition upon circulating adipokines and pro-inflammatory cytokines.
3. Characterise the effect of aromatase inhibition upon relevant mRNA transcript levels in the subcutaneous adipose compartment.

To address these aims, I undertook a double-blind randomised balanced crossover study of pharmacological aromatase inhibition in healthy male volunteers utilising hyperinsulinaemic eugycaemic clamp studies (with stable isotope tracers), measurement of adipokines and pro-inflammatory cytokines in plasma, and assessment of mRNA transcript levels in subcutaneous adipose tissue biopsies.

5.3 Methods

5.3.1 Study Title and Ethical Approvals

Study title: The metabolic effects of aromatase inhibition

Principal Investigator: Dr Fraser W Gibb

Ethical approval was gained from the Lothian Research Ethics Committee in September 2007 (LREC number 07/S1101/03). NHS Lothian granted research and Development approval in August 2007 (R&D number 2007/R/END/03). The study was carried out at the Wellcome Trust Clinical Research Facility at the Western General Hospital, Edinburgh.

5.3.2 Materials

Full details of materials are provided in chapter 2.

Fasting lipid profile, plasma glucose, liver function tests and urea and electrolytes were measured at the biochemistry laboratory of the Royal Infirmary of Edinburgh.

The candidate undertook all of the clinical measurements and sample collection, and gained experience with all of the laboratory assay techniques, but as a result of samples being analysed after the end of his PhD fellowship, he is grateful for the

assistance in completion of qPCR by Karen French and Kerry McInnes, of GCMS by Sanjay Kothiya, and of plasma biochemistry analyses by Rita Upreti

5.3.3 Study design

The design was a double-blind randomised balanced crossover study in healthy male volunteers.

5.3.3.1 Subjects: recruitment, inclusion and exclusion criteria

Recruitment took place between March 2008 and October 2009. Advertisements were placed in local newspapers and posters displayed across the University of Edinburgh, NHS Lothian and other public areas. 20 individuals proceeded through the baseline screening visit but 3 subjects dropped out prior to randomisation. The initial screening visit involved a brief clinical assessment, principally to ensure inclusion and exclusion criteria were satisfied.

Inclusion criteria:

- Men aged between 18 – 65 years
- Normal screening blood tests (urea & electrolytes, liver function tests, lipid profile, thyroid function tests and full blood count)

Exclusion criteria:

- Any significant illness
- Regular medication
- Abnormal screening blood tests (as described above)
- Alcohol excess (defined as greater than 28 units per week)
- Inability to give informed consent

All screened volunteers (n=20) fulfilled the inclusion and exclusion criteria, although three individuals elected not to participate further following the screening visit.

5.3.3.2 Study protocol

Subjects were randomised to initially receive either 1mg anastrozole once daily or identical placebo capsules once daily for 6 weeks (Tayside Pharmaceuticals, Dundee, UK), at the end of which they attended the clinical research facility for a hyperinsulinaemic euglycaemic clamp study (described in detail in section 5.3.5). After a 2 week 'washout' period, subjects 'crossed-over' to the opposite treatment allocation for six weeks, followed by a further clamp study (summarised in figure 5.1). Randomisation codes were generated by one of the project supervisors (RA) and the study was only un-blinded to the principal investigator following completion of analyses.

Subjects who were willing to have a subcutaneous adipose tissue biopsy performed (n=6), attended on a morning separate to the clamp study, within a five-day period

prior to completion of their course of study medication. Paired adipose biopsies (*i.e.* for both the placebo and anastrozole phases) were collected in all six of these individuals.

5.3.3.3 Clinical measurements

Systolic and diastolic blood pressure and pulse rate were measured, after sitting for at least 10 minutes, using a 705IT automatic blood pressure monitor (OMRON Healthcare, Netherlands). Weight and height were obtained in all subjects. Body fat percentage was measured by electrical bioimpedance using a handheld OMRON BF306 Body Fat Monitor (OMRON Healthcare, Henfield, UK).

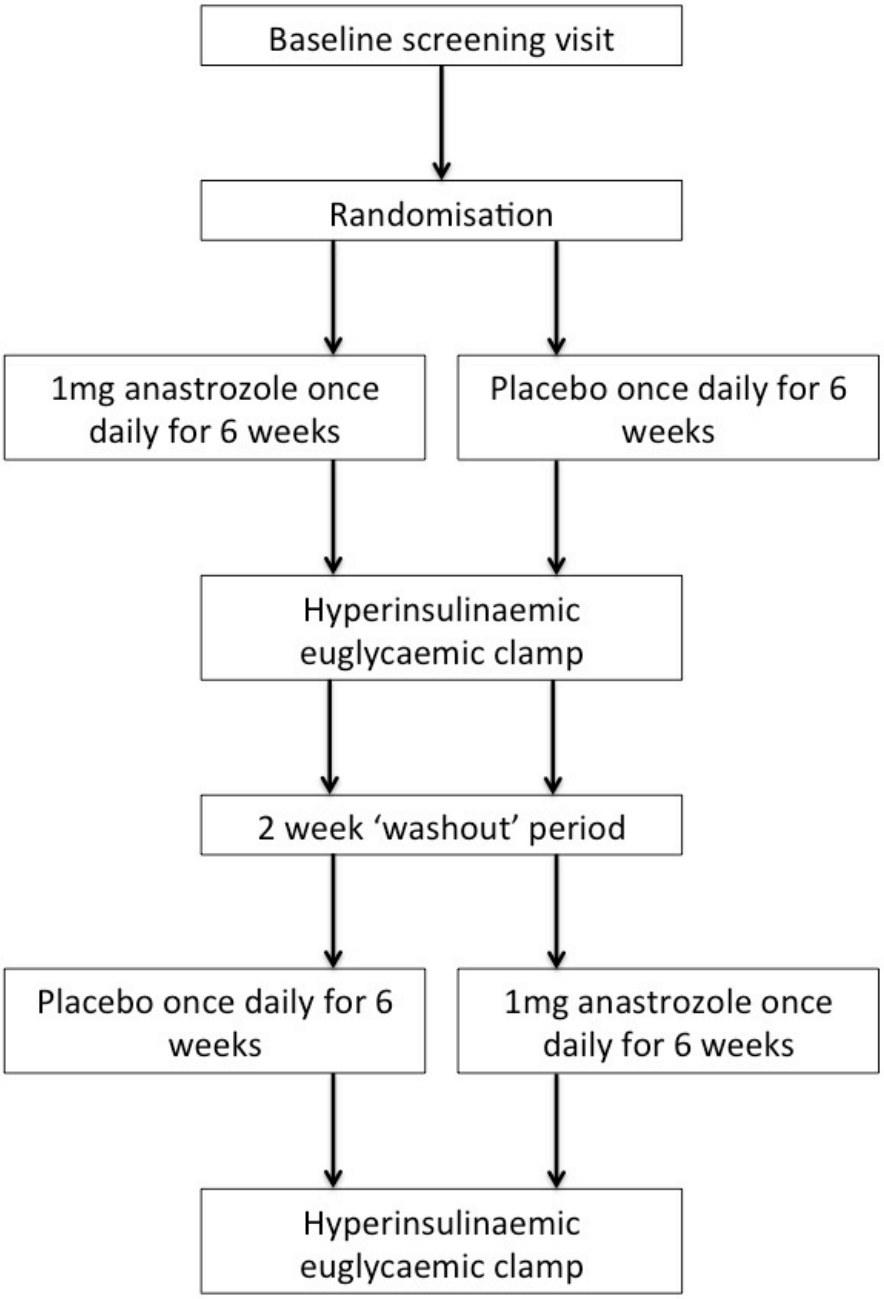


Figure 5.1 Summary of study protocol

5.3.4 Adipose tissue biopsy

Paired subcutaneous adipose biopsies were performed (as described in section 2.2.2) within the final 5 days of both the anastrozole and placebo phases. To summarise, following subcutaneous administration of 5mL local anaesthetic (5ml 2% lidocaine, Hameln Pharmaceuticals, Gloucester, UK), a 19G needle was inserted approximately 10cm lateral to the umbilicus and subcutaneous adipose tissue aspirated by vacuum. The samples were collected in sterile eppendorf tubes, stored immediately in dry ice before transfer to -80°C refrigeration. Adipose tissue was later processed and RNA extracted, as described in detail in section 2.4.3. Following reverse transcription (described in section 2.4.5), real time PCR was performed to analyse the mRNA transcript levels of a battery of genes related to adipogenesis, steroid metabolism and adipocytokines (described in section 2.4.6).

5.3.5 Hyperinsulinaemic euglycaemic clamp with deuterated glucose and glycerol tracers

5.3.5.1 Drug preparation and dosage calculations

Actrapid® insulin was prepared in 0.9% saline at a concentration of 0.3 U/mL. Infusion rates for insulin were based on body surface area. The rate of infusion was calculated as follows:

$$\text{Rate (mL/min)} = \frac{\text{(10 [low dose] or 40 [high dose]) x BSA x 60}}{\text{1000 x 0.3}}$$

Stable isotope tracers were prepared by Dr Alistair Millar (Radiopharmacist, Royal Infirmary of Edinburgh), using water as diluent. 1,1,2,3,3-$^{2}H_5$-glycerol (d5-glycerol) was provided in 8.5 mL vials (40 mg/mL) and 6,6,-$^{2}H_2$-glucose (d2-glucose) in 5 mL vials (350 mg/mL).

Final preparation was performed on the morning of administration, when both glucose and glycerol tracers were diluted in 0.9% saline. Priming boluses of tracer were prepared by addition of 0.5 mL (20 mg) d5-glycerol to 19.5 mL 0.9% sodium chloride and 1 mL (350 mg) d2-glucose to 19 mL 0.9% sodium chloride, giving concentrations of 1 mg/mL and 17.5 mg/mL, respectively. For continuous infusion, d5-glycerol (7.5 mL, 300mg) and d2-glucose (3.2 mL, 1.12g) were added to make up a total volume of 500 mL. Administration of tracers is described in detail in section 5.3.5.2.

5.3.5.2 Clamp protocol

Clamp studies were performed, at completion of both the placebo and anastrozole phases of the study, for assessment of insulin sensitivity (protocol summarised in figure 5.1). Subjects attended the clinical research facility at 07:30 in a fasted state (from 22:00 the preceding evening). Subjects were also asked to abstain from alcohol and tobacco on the day prior to the clamp study. Upon arrival, two venous cannulae were inserted for infusions and a third cannulae was retrogradely inserted in a

contralateral hand vein for sampling of arterialised blood for glucose measurements. Hand veins were 'arterialised' by external heating with an electric blanket throughout the clamp study. The study can be separated into three 90-minute periods:

1. 0 – 90 minutes: Administration of priming boluses of stable isotope tracers and subsequent infusion (as described in section 5.3.5.1). Priming boluses of d2-glucose (17 μmol/kg) and d5-glycerol (1.6 μmol/kg) were administered over 1 minute to attain earlier steady state conditions. Thereafter, continuous infusion of d2-glucose (0.22 μmol/kg/min) and d5-glycerol (0.11 μmol/kg/min) was commenced and maintained at this rate through the full clamp protocol.
2. 90 – 180 minutes: Low dose insulin. An infusion of Actrapid® insulin (Novo Nordisk, Denmark) was commenced at a rate of 10 mU/m^2/min with a parallel infusion of 20% dextrose, adjusted every 5 minutes, to maintain euglycaemia. The low dose period was designed to assess inhibition of lipolysis and suppression of hepatic glucose output.
3. 180 – 270 minutes: High dose insulin. For the final period, the insulin infusion rate was increased to 40 mU/m^2/min, with continuing adjustment of the 20% dextrose infusion rate. The high dose period was designed to assess peripheral glucose uptake.

During the final 15 minutes of each period, 4 blood samples were collected (at 5 minute intervals) in serum gel tubes. These steady state samples were used to measure plasma tracer and tracee concentrations (as described in chapter 2.7). Throughout the insulin infusion (periods 2 and 3), blood glucose levels were clamped

between 4.5 to 5.5 mmol/L, with glucose measurement taking place at 5 minute intervals, from arterialised whole blood, using a glucometer (Accu-Check® Advantage, Roche, Germany).

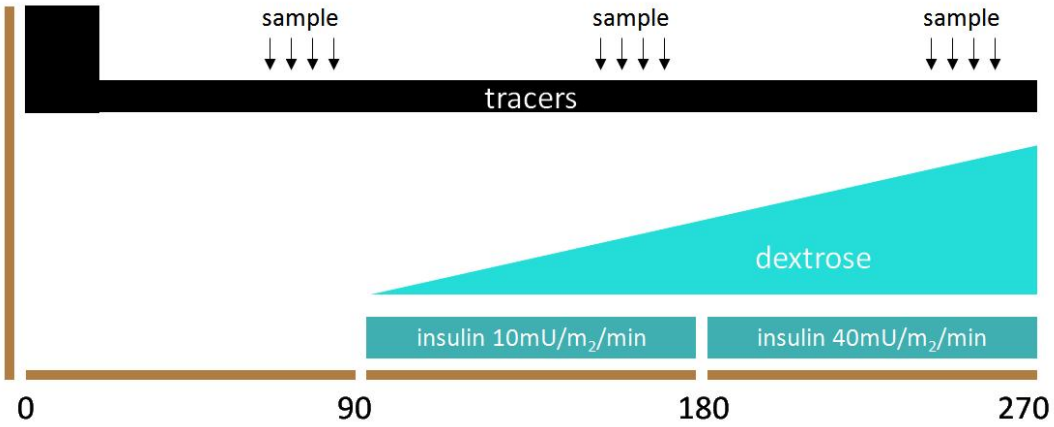


Figure 5.2 Schematic of hyperinsulinaemic euglycaemic clamp, showing period 1 (0 to 90 minutes; no insulin), period 2 (90 to 180 minutes; low dose insulin) and period 3 (180 to 270 minutes; high dose insulin). Blood sampling was performed in the final 15 minutes of each period, as represented by arrows. 20% dextrose infusion was adjusted to maintain euglycaemia in periods 2 and 3.

5.3.5.3 Sample collection

Following insertion of an intravenous cannula, baseline blood samples were taken at approximately 07:30. All blood tubes were S-Monovette® (Sarstedt, Germany) with serum gel 7.5 mL tubes to collect serum, 5.5 mL lithium heparin tubes to collect plasma and 2.5 mL fluoride gel tubes for glucose samples. Glucose samples (fluoride tubes) were sent directly to the Western General Hospital biochemistry laboratory; all other samples were processed in the Clinical Research Facility and stored for future analysis.

All blood samples were centrifuged on a Sigma 4K14 instrument (Munich, Germany) under the following conditions: 1912 g, 10 minutes, 4°C. The resultant plasma / serum was stored at -80°C.

5.3.5.4 Gas-Chromatography Mass Spectrometry

Plasma glucose, glycerol and their respective tracers were quantified by GCMS as described in detail in chapter 2.

5.3.6 Data analysis

5.3.6.1 Calculations

Body mass index was calculated as:

$$\text{BMI } (kg/m^2) = \frac{\text{weight (kg)}}{(\text{height (m)})^2}$$

Body surface area (BSA) was calculated using the Mosteller method (Mosteller, 1987):

$$\text{BSA } (m^2) = \sqrt{\frac{\text{Weight (kg) x height (m)}}{3600}}$$

Glucose disposal (M) under steady state conditions was calculated as the mean volume of glucose infused in the final 15 minutes for phases 2 and 3 of the tracer study (low dose insulin and high dose insulin, respectively) and reported in mg/kg/min.

Rate of appearance (Ra) [glucose and glycerol] and rate of disappearance (Rd) [glucose] were calculated as follows,using the tracer:tracee ratio (TTR), where tracer refers to the isotopically labelled glucose and glycerol, whilst tracee refers to

unlabelled (endogenous) glucose and glycerol. The ratio is derived from the peak areas on the mass spectrum for the respective isotopomers.

For Ra glucose:

$$\frac{Tracer}{Tracee} = T/Tr = \frac{Ra\ d2glucose}{Ra\ glucose}$$

Therefore:

$$Ra\ Glucose = \frac{Ra\ d2Glucose}{T/Tr}$$

Ra = Rd at steady state

The rate of tracer infusion corresponds to the rate of appearance of labelled glucose and glycerol:

Ra d2-Glucose	=	4mg/kg/hour	=	0.067mg/kg/min
Ra d5-Glycerol	=	0.64mg/kg/hour	=	0.01mg/kg/min

The same calculation is used to determine the Ra for glycerol, which equates to the rate of lipolysis, assuming that triglyceride breakdown is the source of unlabelled glycerol.

As a consequence of naturally occurring glucose isotopomers in both the variable rate glucose and d2-glucose infusions, adjustments are required to account for this in the Ra d2-glucose and glucose infusion rate (GIR). The true abundance of pure d2-glucose in the tracer infusion was 92.5%, with the remaining 7.5% containing other naturally occurring isotopic species, such as ^{13}C. Similarly 1.1% of the variable rate glucose infusion was composed of naturally occurring d2-glucose. The following calculation takes this variation into account:

True Ra d2-glucose = 92.5% rate of d2-glucose infusion + 1.1% variable GIR

= (0.0925 X 0.067) + (0.011 x GIR)

= 0.062 + (0.011 x GIR)

Rate of endogenous glucose production (EGP) was calculated using steady state values for Ra glucose. Ra glucose comprises both EGP and the infusion of variable rate unlabelled glucose. An identical 92.5% correction for unlabelled glucose is also required, taking into account the fact that 7.5% of this infusion is composed of other mass isomers, including d2-glucose and ^{13}C species. EGP is calculated thus:

EGP = Ra glucose - (0.925 x GIR)

No correction was required for glycerol, as no exogenous infusion was administered and the abundance of isotopic species was negligible.

5.3.6.2 Statistical analysis

All statistical analyses were undertaken using IBM SPSS for Windows (Version 19.0, Armonk, NY: IBM Corp). Data are presented as mean ± SEM unless otherwise stated. Comparisons between the anastrozole and placebo phases were made using paired Student's t-tests. When data were not normally distributed, logarithmic transformation was undertaken. Where transformation did not result in normally distributed data, non-parametric analyses were undertaken, as detailed in the results section.

Statistical significance was accepted at $p < 0.05$.

The intention was to recruit 20 participants, which was calculated to have 80% power to detect a 15% difference in rates of glucose disposal and appearance, based upon similar studies undertaken in the supervisors' laboratory (Sandeep *et al.* 2005). Unfortunately, due to difficulties in recruitment, only 17 subjects completed the study.

5.4 Results

5.4.1 Subject characteristics

17 individuals completed the study protocol, including 6 who consented to paired subcutaneous adipose biopsies. The first treatment phase was anastrozole in nine subjects and placebo in the remaining eight. The mean age of volunteers was 27.7 ± 2.5 years (range 18 – 50 years). No participants were on any regular medication or suffered from any significant co-morbidity.

5.4.2 Blood pressure

Anastrozole was associated with a 4.7 mmHg increase in systolic blood pressure (138 ± 3 vs. 134. ± 3 mmHg, $p < 0.05$) although no significant difference was observed in diastolic blood pressure (79 ± 2 vs. 78 ± 2 mmHg, $p=0.847$). Heart rate was significantly lower during anastrozole treatment (66 ± 3 vs. 71 ± 3 bpm, $p < 0.05$).

5.4.3 Weight and body composition

No significant differences were noted in weight between the aromatase inhibitor and placebo phases of the study (82.2 ± 3.4 vs. 81.8 ± 3.4 kg, $p=0.404$). Similarly, no differences were observed with respect to percentage body fat (16.4 ± 1.9 vs. 16.4 ± 1.9 %, $p=0.957$) and BMI (25.9 ± 1.1 vs. 25.7 ± 1.1 kg/m^2, $p=0.445$).

5.4.4 Effect of aromatase inhibition upon circulating sex steroid concentrations

Sex steroids were measured in 14 of the 17 subjects, subject to the availability of sufficient serum sample (Table 5.1). Serum estradiol fell in all 14 subjects during aromatase inhibitor therapy, although in one case this difference was negligible. Serum estrone did not fall in 2 of 14 subjects during aromatase inhibitor therapy. It would be reasonable to infer that the single subject with no appreciable change in either estrone or estradiol was not compliant with the study medication. No significant differences were observed in either testosterone or androstenedione concentration between study phases.

	Anastrozole	Placebo	p
Estradiol (pmol/L)	53.7 ± 2.2	64.5 ± 1.7	< 0.001
Estrone (pmol/L)	28.3 ± 1.5	35.3 ± 1.9	< 0.001
Testosterone (nmol/L)	19.4 ± 0.5	19.7 ± 0.6	0.777
Androstenedione (nmol/L)	4.5 ± 0.3	4.5 ± 0.2	0.845

Table 5.1 Serum sex steroid hormone concentrations during anastrozole and placebo phases of the study. Data are presented as mean ± SEM and compared with paired Student's t-test.

5.4.5 Clamped glucose levels, insulin concentration and glucose tracer: tracee ratios (TTR)

Plasma glucose was clamped with a target of 4.5 – 5.5 mmol/L being achieved (mean glucose 5.2 ± 0.03 mmol/L) (Figure 5.3). Achieved plasma insulin concentrations were 3.3 ± 0.7 mU/L, 11.6 ± 1.1 mU/L and 43.6 ± 2.2 mU/L in steady state samples at baseline, low dose and high dose clamps, respectively. No significant differences were observed in insulin concentration between the placebo and anastrozole phase at baseline and during high dose insulin infusion; there was a non-significant trend towards lower insulin concentration during the low dose insulin infusion in the anastrozole phase (Table 5.2). TTRs at each stage of the clamp are shown in figure 5.4. d2-Glucose: glucose ratios achieved steady state during each sampling period. TTR decreased, as expected, during the high dose insulin phase, in concert with higher glucose infusion rates.

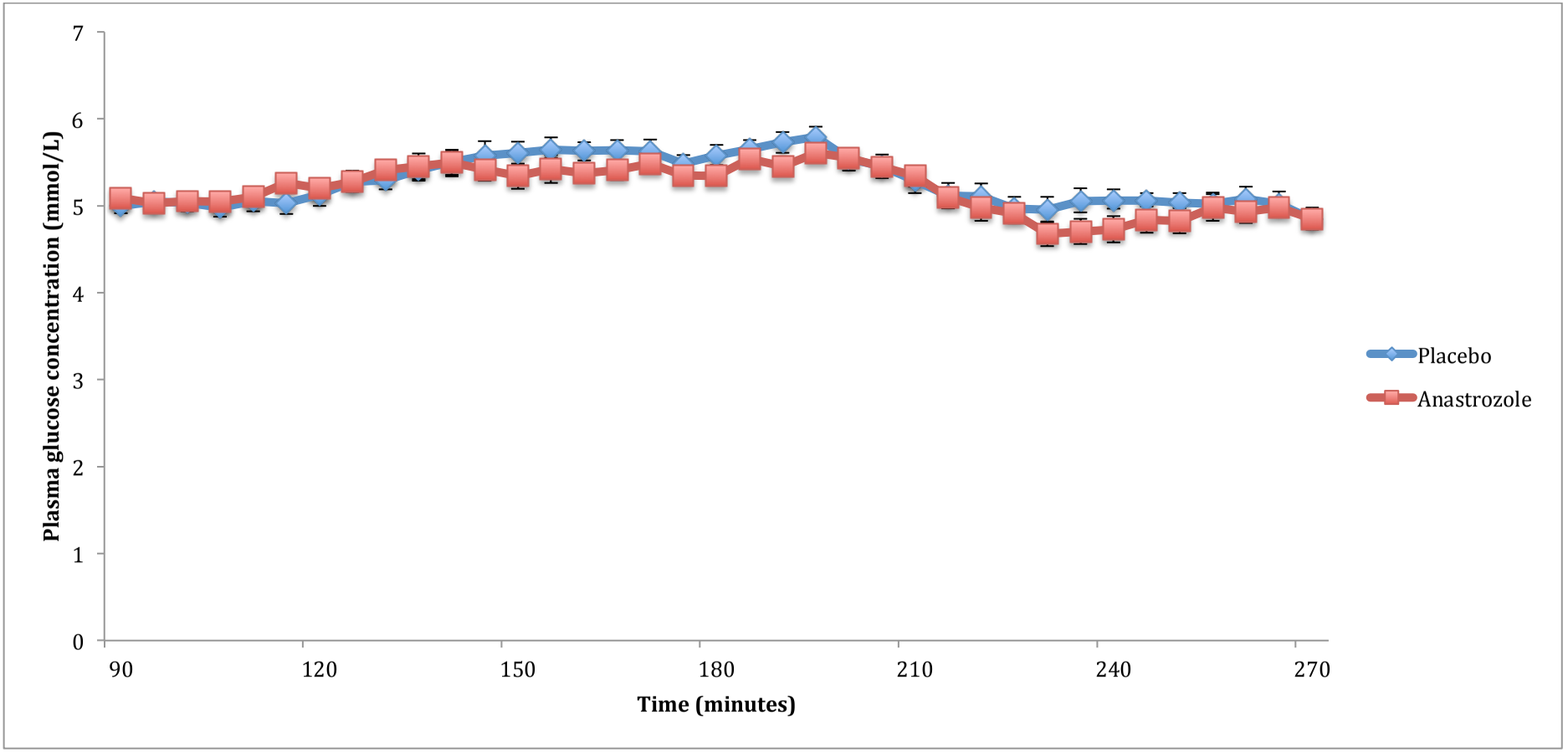


Figure 5.3 Plasma glucose concentrations (mmol/L) across hyperinsulinaemic euglycaemic clamp during either placebo or anastrozole treatment. Data are mean; error bars represent SEM.

Time (minutes)	Anastrozole	Placebo	p
80	3.0 ± 0.9	3.5 ± 1.0	0.551
170	9.8 ± 1.4	13.4 ± 1.6	0.066
260	43.6 ± 3.0	43.8 ± 3.3	0.916

Table 5.2 Achieved plasma insulin concentration (mU/L) across the course of clamp studies. Data are expressed as mean ± SEM and compared by paired Student's t-tests.

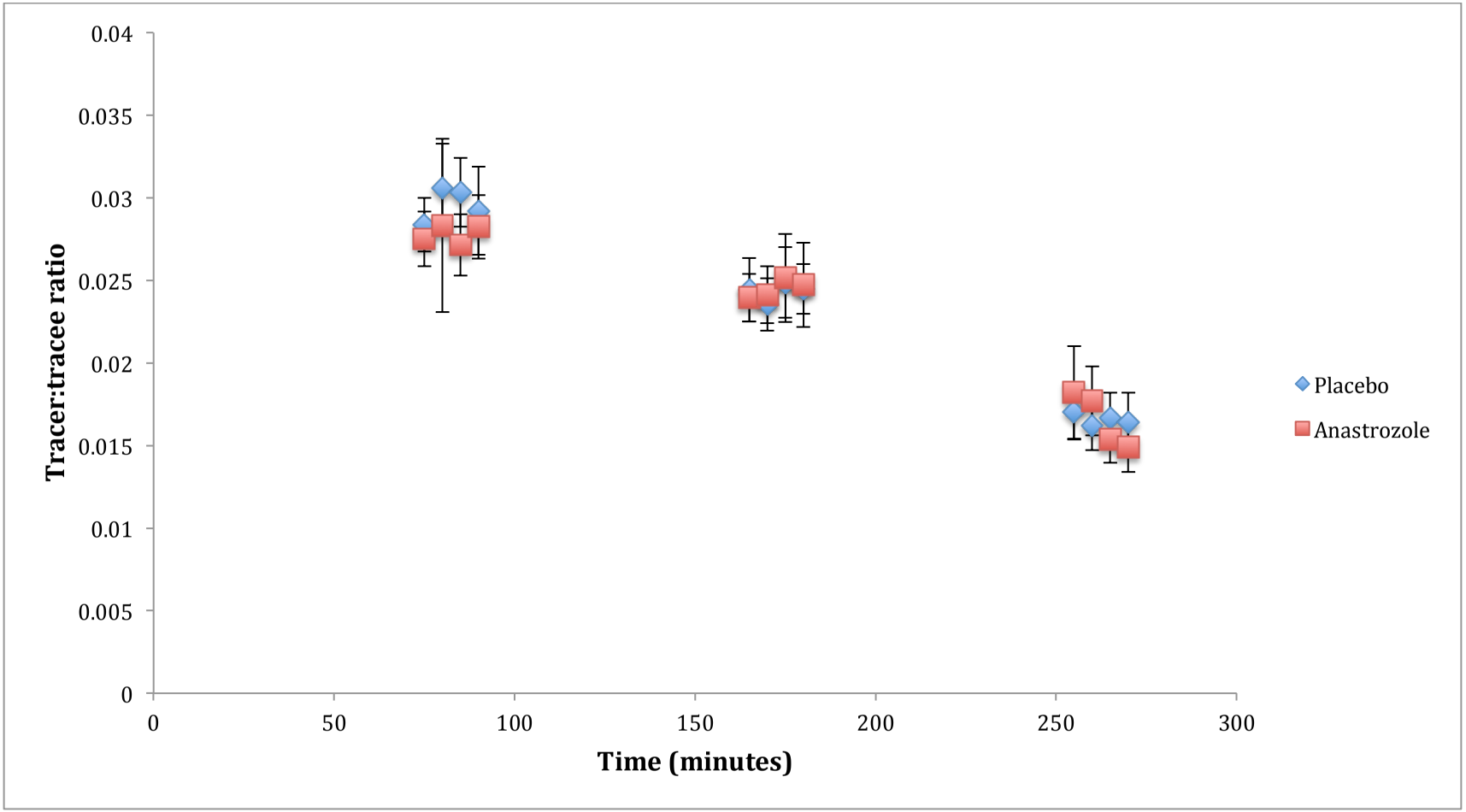


Figure 5.4 d2-Glucose tracer:tracee ratios during the hyperinsulinaemic euglycaemic clamp in both the anastrozole and placebo phases. Presented as mean ± SEM.

5.4.6 Whole body glucose disposal

Whole body glucose disposal is inferred from the glucose infusion rate (M) during the high dose insulin phase of the clamp. Glucose infusion rates throughout the clamp studies are represented in figure 5.5. No difference in M value was noted between anastrozole and placebo phases during the high dose clamp. However a significantly higher M value was observed in the placebo phase during the low dose clamp (Table 5.3). M values were also corrected for fat free mass (FFM), which largely represents skeletal muscle, the main site of glucose uptake; this did not materially affect the results.

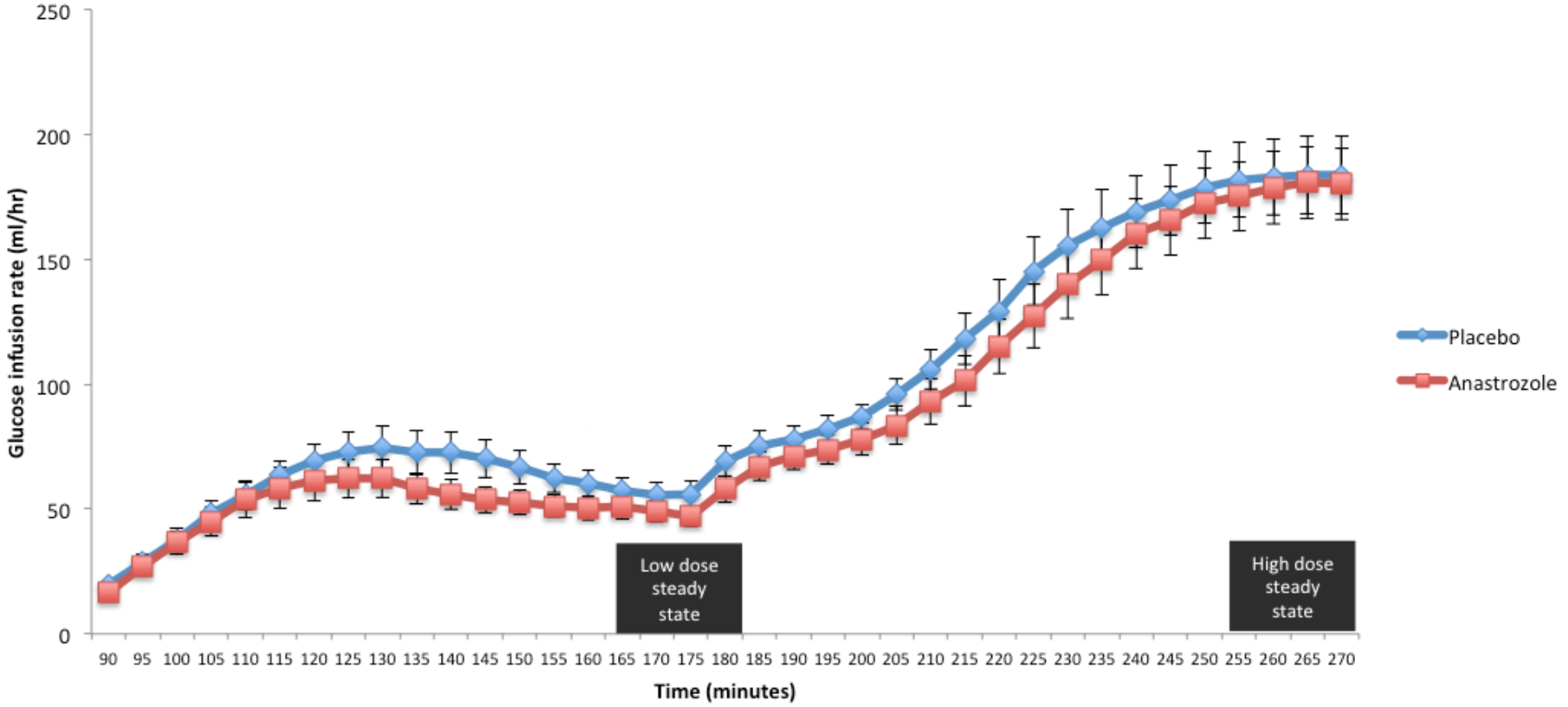


Figure 5.5 Glucose infusion rate during the low and high dose phases of the hyperinsulinaemic clamp study. Data are presented as mean ± SEM.

	Anastrozole	Placebo	p
Glucose infusion rate – Low dose clamp (mg/kg/min)	2.19 ± 0.24	2.55 ± 0.28	0.024
Glucose infusion rate – Low dose clamp (mg/kgFFM/min)	2.57 ± 0.25	2.99 ± 0.27	0.025
Glucose infusion rate – High dose clamp (mg/kg/min)	7.53 ± 0.66	7.77 ± 0.75	0.599
Glucose infusion rate – High dose clamp (mg/kgFFM/min)	8.87 ± 0.71	9.14 ± 0.80	0.626

Table 5.3 Glucose infusion rates (M) during low and high dose insulin phases of clamp studies. Presented as mean ± SEM and compared by paired Student's t-tests.

5.4.7 Rate of glucose disposal (Rd glucose)

Rd glucose is derived from the M value and TTR; it permits adjustment for residual hepatic glucose production, as well as glucose infusion. The results of Rd glucose during low dose insulin were congruent with previously reported M values, namely a trend towards decreased glucose disposal associated with anastrozole. Of greater significance was the recognition of a 23.7% decrease in glucose disposal rate during the high dose insulin phase, in those receiving anastrozole (Table 5.4).

	Anastrozole	Placebo	p
Tracer only (μmol/kg/min)	7.77 ± 0.56	7.50 ± 0.46	0.271
Tracer only (μmol/kgFFM/min	9.35 ± 0.58	9.04 ± 0.60	0.313
Low-dose insulin (μmol/kg/min)	15.04 ± 1.13	16.46 ± 1.50	0.053
Low-dose insulin (μmol/kgFFM/min)	16.79 ± 1.26	18.28 ± 1.47	0.068
High-dose insulin (μmol/kg/min)	38.52 ± 7.73	47.61 ± 5.25	0.039§
High-dose insulin (μmol/kgFFM/min)	38.56 ± 7.73	50.50 ± 5.40	0.039§

Table 5.4 Total Rd glucose across hyperinsulinaemic euglycaemic clamp presented as mean ± SEM with and without adjustment for fat free mass (FFM) and compared by paired Student's t-tests. * Log transformed to normalise distribution. § Compared by Related-Samples Wilcoxon Signed Rank Test, as non-parametric tests deemed more appropriate due to lower accuracy of TTR values during high-dose insulin phase.

5.4.8 Endogenous glucose production

Endogenous glucose production (EGP) in the fasting state prior to insulin infusion is equivalent to Rd glucose. EGP was calculated during low dose insulin, and was not affected by anastrozole. No significant difference in EGP was noted between the placebo and anastrozole phases of this study (Table 5.5). EGP is not reported for the final clamp period, as complete suppression of EGP is achieved during high dose insulin infusion.

	Anastrozole	Placebo	p
EGP: Tracer only (μmol/kg/min)	7.77 ± 0.46	7.50 ± 0.46	0.271
EGP: Tracer only (μmol/kgFFM/min)	9.35 ± 0.58	9.04 ± 0.60	0.313
EGP: Low-dose insulin (μmol/kg/min)	3.79 ± 1.01	3.37 ± 1.10	0.562
EGP: Low-dose insulin (μmol/kgFFM/min)	5.55 ± 1.12	5.19 ± 1.25	0.652
Suppression glucose production: Low-dose insulin (%)	61.21 ± 13.02	64.96 ± 12.47	0.723§
Suppression glucose production: Low-dose insulin (%) [corrected for FFM]	49.75 ± 11.50	51.97 ± 11.32	0.981§

Table 5.5 Endogenous glucose production (EGP) during first two phases of hyperinsulinaemic euglycaemic clamp. Presented as mean ± SEM compared by paired Student's t-tests. § Data not normally distributed despite transformation and therefore compared by Related-Samples Wilcoxon Signed Rank Test.

5.4.9 Glycerol concentrations during clamp

As expected, glycerol concentration fell progressively across the low and high dose phases of the clamp studies, as a consequence of reduced lipolysis. No significant differences were detected between the anastrozole and placebo phases of the study (Table 5.6).

	Anastrozole	Placebo	p
Tracer only	62.3 ± 5.1	66.4 ± 4.7	0.223
Low-dose insulin	35.5 ± 5.2	38.1 ± 4.0	0.388
High-dose insulin	27.9 ± 5.0	32.1 ± 3.9	0.086
% suppression from baseline to low dose clamp	52.8 ± 4.3	57.3 ± 3.4	0.426
% suppression from baseline to high dose clamp	40.8 ± 4.2	46.8 ± 3.7	0.071

Table 5.6 Plasma glycerol concentrations (μmol/L) across the hyperglycaemic euglycaemic clamp (mean of four steady state values). Presented as mean ± SEM and compared by Paired-samples Student's t-tests.

5.4.10 Tracer: Tracee Ratios for d5-glycerol: glycerol

Steady state values were obtained for d5-glycerol: glycerol Tracer: tracee ratios (TTR) across the clamp studies, as demonstrated in figure 5.6. TTRs increased across the course of clamp studies, reflecting the expected fall in endogenous glycerol.

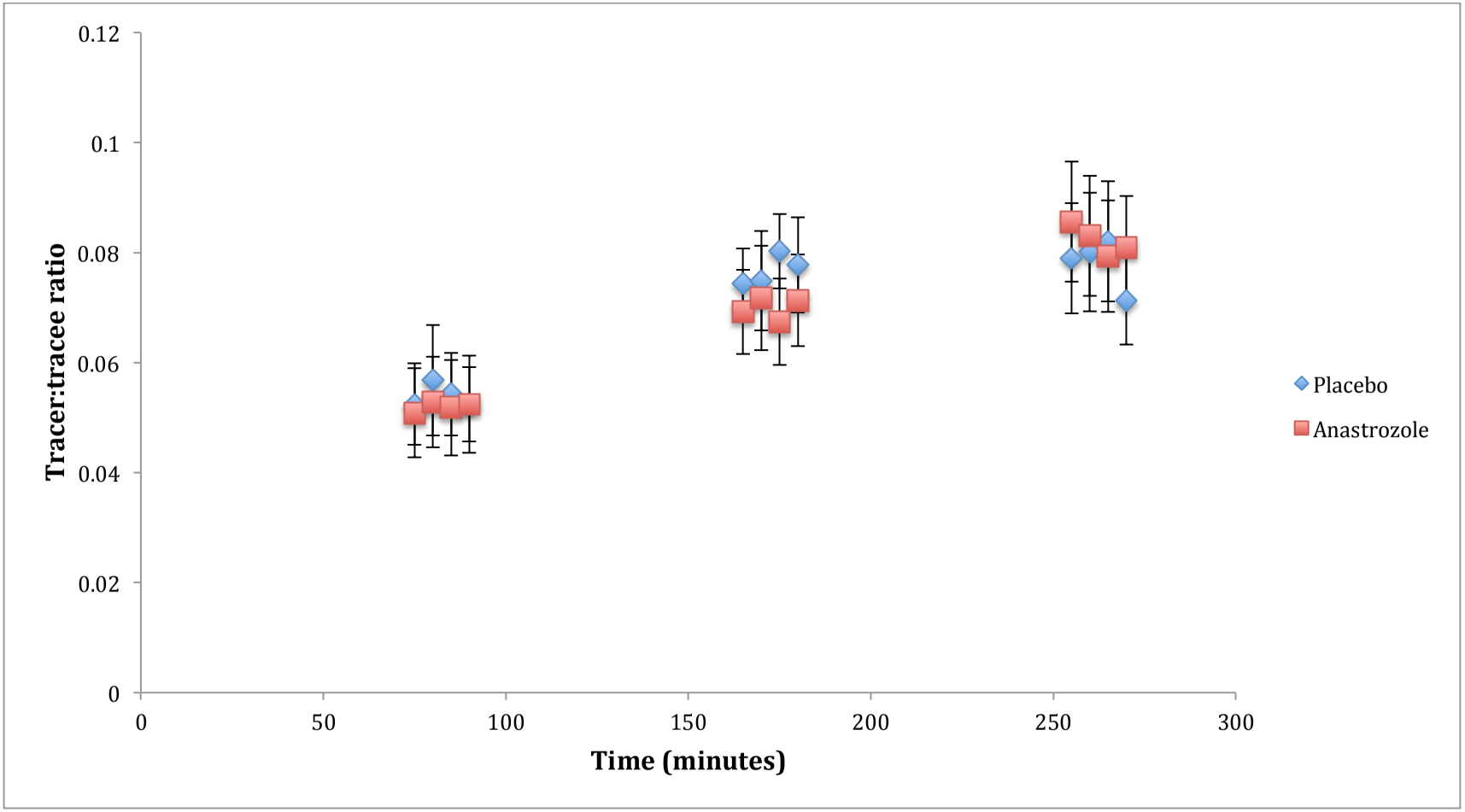


Figure 5.6 d5-Glycerol tracer:tracee ratios during the hyperinsulinaemic euglycaemic clamp in both the anastrozole and placebo phases. Presented as mean ± SEM.

5.4.11 Glycerol kinetics

Rate of appearance for glycerol (Ra glycerol) was not altered by anastrozole, as summarised in table 5.7. Correction for fat free mass did not significantly influence these results.

	Anastrozole	Placebo	p
Ra glycerol: Tracer only (μmol/kg/min)	2.85 ± 0.36	2.79 ± 0.34	0.777
Ra glycerol: Tracer only (μmol/kgFFM/min)	3.39 ± 0.43	3.31 ± 0.38	0.704
Ra glycerol: Low-dose insulin (μmol/kg/min)	1.87 ± 0.19	1.73 ± 0.17	0.287
Ra glycerol: Low-dose insulin (μmol/kgFFM/min)	2.23± 0.23	2.05 ± 0.19	0.260
Ra glycerol: High-dose insulin (μmol/kg/min)	1.69 ± 0.18	1.77 ± 0.20	0.525
Ra glycerol: High-dose insulin (μmol/kgFFM/min)	2.03 ± 0.22	2.11 ± 0.24	0.594
% suppression of glycerol Ra from base to low dose	28.0 ± 4.6	32.0 ± 4.5	0.302
% suppression of glycerol Ra from base to high dose	34.3 ± 5.2	31.9 ± 5.6	0.609

Table 5.7 Ra glycerol and % suppression of glycerol Ra across hyperinsulinaemic euglycaemic clamp. Data presented as mean ± SEM and compared by paired Student's t-tests.

5.4.12 Lipid profile

Anastrozole treatment resulted in a 6.4% reduction in total serum cholesterol ($p < 0.05$). This was a consequence of non-significant trends towards lower HDL cholesterol (7.4%) and LDL cholesterol (6.6%). No significant differences in either triglyceride or Cholesterol:HDL ratio were observed. Full results are summarised in table 5.8.

	Anastrozole	Placebo	p
Total cholesterol (mmol/L)	3.86 ± 0.13	4.12 ± 0.14	0.041
HDL cholesterol (mmol/L)	1.01 ± 0.05	1.09 ± 0.05	0.080
LDL cholesterol (mmol/L)	2.39 ± 0.14	2.56 ± 0.14	0.118
Triglyceride (mmol/L)	1.02 ± 0.09	1.05 ± 0.16	0.770
Total cholesterol:HDL ratio	3.99 ± 0.23	3.96 ± 0.25	0.816

Table 5.8 Effect of anastrozole therapy on circulating lipid profile presented as mean ± SEM. Compared by paired-samples Student's t-tests.

5.4.13 Adipokines and pro-inflammatory cytokines

Serum leptin was reduced by 28% following 6 weeks of anastrozole administration. No significant differences were noted in any of the other adipokines and cytokines measured (Table 5.9)

	Anastrozole	Placebo	p
Leptin (pg/mL)	4249 ± 999	5890 ± 1349	0.039§
Adiponectin (μg/mL)	11.7 ± 4.7	14.6 ±2.6	0.188*
Resistin (pg/mL)	32.6 ± 2.9	32.2 ± 2.8	0.830
IL-8 (pg/mL)	5.6 ± 0.8	6.2 ± 1.4	0.868§
MCP-1 (pg/mL)	226.2 ± 13.4	216.1 ± 10.0	0.362

Table 5.9 Effect of anastrozole therapy on circulating adipokines and pro-inflammatory cytokines presented as mean ± SEM. Compared by paired Student's t-tests (log transformed data*) except where data not normally distributed following log transformation, where Related-Samples Wilcoxon Signed Rank Tests were employed§.

5.4.14 Adipose tissue mRNA

mRNA expression was assessed for genes where current evidence suggested a potential regulatory role for sex steroid hormones. Of 27 genes assessed, only 2 demonstrated changes in expression related to anastrozole (Table 5.10): Estrogen receptor β and perilipin 2, both of which were down-regulated by anastrozole (45.1% and 8.8%, respectively).

	Anastrozole	Placebo	p
Steroid hormone synthesis and metabolism			
HSD11B1 11βHSD1	1.215 ± 0.388	1.202 ± 0.356	0.959
CYP19A1 Aromatase	1.042 ± 0.269	0.961 ± 0.320	0.848
Steroid hormone receptors			
AR Androgen receptor	1.264 ± 0.113	1.332 ± 0.108	0.650
ESR1 Estrogen receptor α	1.064 ± 0.172	0.885 ± 0.061	0.398
ESR2 Estrogen receptor β	0.538 ± 0.276	0.979 ± 0.344	**0.040**
Adipogenesis, lipogenesis and lipolysis			
ACACA Acetyl CoA carboxylase	1.097 ± 0.207	1.169 ± 0.297	0.775
UCP2 Uncoupling protein 2	1.108 ± 0.029	1.198 ± 0.140	0.474
FASN Fatty acid synthase	0.911 ± 0.281	0.938 ± 0.339	0.807
LIPE Hormone sensitive lipase	1.080 ± 0.296	0.906 ± 0.262	0.223
LPL Lipoprotein lipase	1.093 ± 0.109	1.053 ± 0.114	0.750
PLIN2 Perilipin 2	1.069 ± 0.094	1.172 ± 0.101	**0.045**
CTNNB1 β-catenin	1.121 ± 0.072	1.055 ± 0.089	0.653
PNPLA2 Adipose triglyceride lipase	0.763 ± 0.106	0.763 ± 0.115	0.999
PPARG Peroxisome proliferator-activated receptor γ	1.098 ± 0.171	1.077 ± 0.119	0.855
PPARGC1A PGC-1α	1.267 ± 0.172	1.043 ± 0.289	0.270
Lipid and sterol metabolism			
CETP Cholesterol ester transfer protein	0.909 ± 0.347	0.890 ± 0.420	0.953
SREBF1 Sterol regulatory element binding transcription factor 1	0.979 ± 0.256	0.914 ± 0.261	0.660

	Anastrozole	Placebo	p
SREBF2 Sterol regulatory element binding transcription factor	1.108 ± 0.029	1.198 ± 0.140	0.150
HMGCR HMG CoA Reductase	0.788 ± 0.089	0.915 ± 0.130	0.529
HMGCS1 HMG CoA Synthase (soluble)	1.012 ± 0.044	0.881 ± 0.058	0.083
LDLR LDL receptor	0.886 ± 0.153	0.995 ± 0.232	0.661
DGAT2 Diacylglycerol O-acyltransferase 2	1.041 ± 0.289	0.883 ± 0.234	0.448
Adipokines			
LEP Leptin	0.959 ± 0.150	1.041 ± 0.175	0.613
ADIPOQ Adiponectin	1.098 ± 0.171	1.077 ± 0.119	0.855
Miscellaneous			
AGT Angiotensinogen	0.829 ± 0.191	0.954 ± 0.263	0.617
ADRA2A α-2-adrenergic receptor	0.871 ± 0.088	1.028 ± 0.211	0.513
ADRB1 β-1-adrenergic receptor	0.861 ± 0.101	1.057 ± 0.241	0.298

Table 5.10 Subcutaneous adipose tissue mRNA transcript levels (relative to cyclophylin) during anastrozole and placebo treatment phases in 6 subjects. Data are presented as mean ± SEM. Analysed by paired Student's t-tests.

5.5 Discussion

Predicting the metabolic effects of aromatase inhibition in men is potentially more difficult than in women; where both estrogen deficiency and relative androgen excess would be expected to exert deleterious effects. In men, the effects of suppressing estrogen are much less clear and, in most prior investigations, have been accompanied by subnormal testosterone concentrations. As noted previously, congenital absence of aromatase activity results in adverse metabolic phenotypes in both mice and men (Simpson *et al.* 1995), however it does not necessarily follow that acquired suppression of aromatase would have similar consequences. In this chapter I have presented a double-blind randomised controlled study designed specifically to address this issue. Consistent with the original hypothesis, I have shown that short-term aromatase inhibition is associated with decreased insulin-stimulated glucose disposal, with no significant effect on hepatic glucose production or lipolysis. No effect on body fat was observed, although serum leptin concentration was lower during aromatase inhibitor therapy. No substantial effect upon mRNA transcript levels was noted in the subcutaneous adipose compartment. Aromatase inhibition was also associated with a significant increase in systolic blood pressure and decreased plasma total cholesterol.

A number of potential populations could have been selected for assessment in this study, including men with late onset hypogonadism (LOH), obesity related hypogonadism or T2DM. Ultimately a decision was made to determine the metabolic effect of aromatase inhibition on healthy volunteers. Whilst there is potentially significant merit in determining whether aromatase inhibition is capable of

ameliorating testosterone deficiency (and its consequences), it is also important to determine whether a shift in the balance of androgen and estrogen action can itself generate a pathological metabolic state in previously healthy men. Recruitment of healthy volunteers for a study which mandated daily adherence to medication for a total of 12 weeks (6 weeks placebo and 6 weeks anastrozole) proved challenging and fell 3 short of the target of 20 subjects. Had recruitment been more straightforward, it would have been preferable to limit the degree of heterogeneity within the study population, with respect to age (which ranged from 18 to 50 years) and also body composition (percentage body fat ranged from 4 to 28%). Age has been identified as a factor which influences the metabolic effects of aromatase inhibitors, albeit in much older men (mean age 76) than the current study (Lapauw *et al.* 2009).

Anastrozole was selected in preference to the other 3rd generation aromatase inhibitors on the basis of its pharmacokinetic profile and superior specificity. Anastrozole has a shorter half-life than letrozole (41 hours vs. 2 – 4 days) and consequently a shorter time to steady state plasma levels (7 days vs. 60 days) (Buzdar *et al.* 2002). These properties were attractive given the relatively short treatment period (6 weeks) and also ensured that sufficient 'wash out' was achieved during the two weeks between placebo and anastrozole phases. Letrozole has been associated with significant reductions in both morning plasma cortisol (Bisagni *et al.* 1996) and ACTH-stimulated cortisol (Bajetta *et al.* 1999), across similar time-scales to that employed in this study (and other studies assessing the metabolic effect of aromatase inhibitors). Cortisol suppression is potentially an important confounder in any study of insulin sensitivity. The relationship between glucocorticoid excess and insulin resistance is well established, however even pharmacological antagonism of the glucocorticoid

receptor in eucortisolaemic individuals is associated with significant changes in glucose metabolism (Garrel *et al.* 1995). Anastrozole is not associated with any changes in plasma cortisol (Buzdar *et al.* 2001) and is therefore ideally suited to purely assess effects related to changes in sex steroid concentration.

The duration of aromatase inhibitor administration in this study (6 weeks) compares favourably to other similar studies, which have been limited to between 1 and 4 weeks (Lapauw *et al.* 2009; Lapauw *et al.* 2010). 6 weeks was selected as a reasonable period to achieve stable plasma levels of anastrozole and observe changes mediated by sex hormone mediated transcriptional regulation; it was likely to have been too short to detect significant changes in body composition and consequent effects on insulin sensitivity.

Aromatase inhibition in post-menopausal women is associated with durable, profound (> 90%) suppression of circulating estradiol (Bajetta *et al.* 2002) but this is not the case in men, where a compensatory increase in luteinizing hormone (LH) mitigates the degree of estradiol suppression. This occurs because feedback at the hypothalamic-pituitary level is principally mediated via estrogen receptor rather than androgen receptor (Raven *et al.* 2006), with consequent increase in LH and testosterone overcoming the aromatase inhibitor 'block'. It is likely that duration of therapy is also relevant, as estradiol suppression was 20% in men treated with anastrozole for 12 months (Burnett-Bowie *et al.* 2009) compared to 62% following 4 weeks of letrozole in a similar study population (Lapauw *et al.* 2009). In this study, estradiol fell by a modest 16.7%, which is significantly less than most other reports in the literature. Only a single subject appeared to achieve no estrogen suppression,

presumably a consequence of non-compliance but this did not significantly skew the mean suppression. Planned measurement of plasma anastrozole concentrations will provide further information with respect to compliance. More surprising than the modest reduction in circulating estrogens, was the absence of any effect upon androgen concentration (testosterone and androstenedione) following six weeks of anastrozole administration. This is difficult to reconcile, as the obvious explanation for limited suppression of estradiol would be a compensatory elevation of LH, and consequently testosterone, following a prolonged period of treatment. Planned measurement of LH will offer clarification, although it would be surprising to observe any significant change, in the context of unaltered testosterone concentration. In mice, anastrozole is significantly less abundant in brain tissue than letrozole (Miyajima *et al.* 2013), which may account for less pronounced excursions in LH observed with anastrozole in human studies; assuming local hypothalamo-pituitary generation of estradiol is more important than availability of circulating estrogen (Burnett-Bowie *et al.* 2009; Raven *et al.* 2006; T'Sjoen *et al.* 2005). However, one week of anastrozole in healthy men did result in substantial elevations in LH (100%) and testosterone (53%) with concomitant suppression of estradiol (50%), albeit using a daily dose ten times greater than the standard dose (1 mg) employed in this study (Hayes *et al.* 2000).

Reported androgen and estrogen results were based on LC MS/MS analysis, regarded as the gold standard for measurement of sex steroid hormones. The superiority of mass spectrometric analysis has been demonstrated in the context of aromatase inhibition, where immunometric methods are prone to significantly underestimate estradiol suppression in post-menopausal women (Stanten *et al.* 2007). The vast

majority of previously reported aromatase inhibitor studies, including all those in men, have relied upon immunoassays, which are known to perform poorly at low estradiol concentrations, where a positive bias is often observed (Huhtaniemi *et al.* 2012). A significant limit in interpreting plasma sex steroid levels is the fact that they do not necessarily reflect tissue concentrations. This has been observed in breast tissue from pre- and post-menopausal women where, despite markedly divergent plasma concentrations, tissue estradiol concentration is equivalent (Pasqualini *et al*. 1996*)* In men and post-menopausal women, local generation and action of estradiol, in adipose tissue and skeletal muscle, is likely to be more physiologically relevant than distant action. In view of the lower than anticipated suppression of serum estradiol in this study, it would be interesting to determine the extent to which anastrozole altered the tissue concentration of sex steroids.

This study is the first in men to achieve suppression of circulating estradiol, whilst maintaining a stable testosterone level, with aromatase inhibitor alone. Another approach designed to deal with LH compensation, is to suppress gonadotrophins with a GnRH agonist and maintain plasma testosterone concentration with transdermal testosterone replacement (Finkelstein *et al.* 2013), permitting a clearer assessment of the relative contributions of estrogens and androgens, although this approach does not mimic physiological daily variation in sex steroid levels. This elegant model suggests that estrogen deficiency, but not androgen deficiency, is associated with insulin resistance, although it is not clear whether this is independent of changes in intra-abdominal fat, which is increased with estrogen deficiency (Joel Finkelstein, personal communication, May 2014). In failing to 'clamp' testosterone levels during aromatase inhibition, as observed in most aromatase inhibitor studies, it can be argued that

metabolic changes may be related to concurrent elevation of testosterone, as much as suppression of estradiol. No evidence exists to suggest supra-physiological testosterone levels are associated with insulin resistance and, indeed, recent work by Finkelstein *et al.* appears to refute a role for testosterone in mediating insulin sensitivity. However, supra-physiological estradiol is associated with reduced muscle GLUT4 expression (Barros *et al.* 2008) and an increased risk of T2DM in women (Ding *et al.* 2006), thereby establishing a possible precedent of deleterious effects associated with excessive sex steroid action.

In this study, body fat percentage was estimated using a hand-held bioelectric impedance meter, an inexpensive but relatively imprecise modality, which provides no additional information regarding compartment specific changes (Fogelholm *et al.* 1997). No difference was detected in weight, BMI, WHR or body fat percentage between anastrozole and placebo phases, which was not unexpected as it may take longer than six weeks to observe sex hormone mediated changes in body composition, particularly with such low sensitivity methodologies. In support of this, 10 weeks of anastrozole therapy (1mg daily) did not result in any change in body composition, as determined by DEXA and calliper measurement, in healthy young men (age 15 – 22 years) (Mauras *et al.* 2000). Despite no obvious change in body composition, in this study, serum leptin concentration was reduced by 28% during the anastrozole phase; consistent with the effect reported previously, following 4 weeks of letrozole administration (Lapauw *et al.* 2009). In the current study, no effect on leptin mRNA expression was observed in subcutaneous adipose tissue, suggesting reduced leptin concentration is not mediated by a direct effect on transcription in the subcutaneous depot. Longer duration of aromatase inhibitor therapy in men (16 weeks) has been

shown to increase body fat percentage, with a disproportionate increase in intra-abdominal fat (Finkelstein *et al.* 2013). Leptin is preferentially secreted by subcutaneous, rather than omental adipocytes (Gottschling-Zeller *et al.* 1999), raising the possibility that the change in leptin concentration, observed in this study, is a consequence of shift from subcutaneous to visceral adipose deposition. This is certainly consistent with the observed increase in insulin resistance but would require verification by more detailed assessment of body fat compartments (*e.g.* single-slice CT). Alternatively, the aromatase inhibitor related reduction in insulin sensitivity observed in this study may be mediated by factors independent of adiposity.

A significant strength of this study was the use of the gold-standard hyperinsulinaemic euglycaemic clamp technique to determine insulin sensitivity (De Fronzo *et al.* 1979). The use of deuterated glucose tracer provides supplemental information regarding the contribution of peripheral and hepatic insulin sensitivity and deuterated glycerol permits assessment of lipolysis. Deuterated glucose was selected, as unlike ^{13}C labelled tracers, the label cannot be recycled via gluconeogenic pathways and therefore provides the most accurate measure of endogenous glucose production (Choukem & Gautier 2008). Deuterated glycerol provides a measure of adipose tissue insulin sensitivity; as there is no re-uptake of glycerol into adipocytes, the rate of appearance of glycerol corresponds to rate of lipolysis. Adjustments for fat free mass were reported, as this is the primary site of glucose uptake, however these adjustments did not materially affect the results. In particularly insulin sensitive individuals, maximal effects of insulin may occur at a lower concentration than is conventionally employed during standard clamp protocols, thus introducing the possibility of missing real differences between groups; this may have been an issue

given the young age and lean body composition of several subjects. Glucose TTR data during the high dose insulin phase were less consistent than during earlier phases, due to the requirement for higher glucose infusion rates, with high dose insulin, and subsequent fall in TTR. This is a relatively common issue and has led to a change in practice, namely the addition of glucose tracer to the variable 20% dextrose infusion fluid. Notwithstanding these technical challenges, this study has provided clear evidence of increased peripheral insulin resistance, manifesting as a 23.7% decrease in glucose disposal, during aromatase inhibitor therapy. No effect on hepatic or adipose insulin sensitivity was detected.

Previous investigators have either failed to demonstrate any aromatase inhibitor effect on insulin sensitivity or have reported improved insulin sensitivity following short-term aromatase inhibition. The first study to suggest improved insulin sensitivity assessed letrozole in 18 healthy men (divided into older and younger age groups) over a 4-week period (Lapauw *et al.* 2009). Due to insufficient washout in the 2-week interval between treatment phases, placebo results were discarded in all subjects who initially received letrozole (n = 10). Improved insulin sensitivity was inferred from changes in fasting glucose and insulin, in the younger subjects only, although no specific indices of insulin sensitivity were reported (*e.g.* HOMA-IR or QUICKI). As alluded to previously, letrozole is known to significantly suppress plasma cortisol levels, however morning cortisol concentrations were not reported in this study, leaving open the possibility that improvements in insulin sensitivity may have been a consequence of cortisol suppression. A subsequent study by the same investigators assessed letrozole effects over a 1-week period either alone (n = 10) or in combination with transdermal estradiol (n = 10) to maintain physiological levels (Lapauw *et al.*

2010). Hyperinsulinaemic euglycaemic clamp studies suggested improved insulin sensitivity in the AI group, but not the AI plus estradiol group, although only after correction for FFM. The same potential criticisms apply regarding the choice of aromatase inhibitor and absence of cortisol measurement. Furthermore, the estradiol replacement group developed subnormal testosterone levels, suggesting supraphysiological estradiol replacement and consequent LH suppression. It is difficult to conclude from these data that estrogen suppression is metabolically beneficial in men; even if this were the case, the net effect of deleterious longer term changes in body composition may be of greater import with respect to insulin sensitivity. 10 weeks of anastrozole in young men and 12 weeks of anastrozole in elderly hypogonadal men did not alter fasting insulin or glucose concentrations (Mauras *et al.* 2000; Dougherty *et al.* 2005). Despite discordance in relation to these earlier studies, insulin sensitivity findings in this thesis are consistent with the outcome of the largest and longest study of anastrozole in men where estrogen deficiency, but not androgen deficiency, was associated with increased insulin resistance (Joel Finkelstein, personal communication, May 2014).

The absence of a demonstrable effect upon subcutaneous adipose tissue mRNA transcription or rudimentary measures of body composition does not, as noted previously, preclude the possibility that changes in insulin sensitivity are mediated, at least in part, by a shift in fat distribution. Nonetheless, given the principal metabolic consequence of aromatase inhibition is peripheral insulin resistance, direct effects upon skeletal muscle are likely to be involved. Aromatase is expressed in skeletal muscle and may generate equivalent quantities of estrogen to adipose tissue, in men and post-menopausal women (Larionov *et al.* 2003; Longcope *et al*. 1978). Pre-

menopausal women are protected from nonesterified fatty acid (NEFA) induced peripheral insulin resistance, compared to age and BMI-matched men (Frias *et al.* 2001); insights from animal models raise the possibility that this difference is estrogen related. Estrogen receptor α-knockout mice (ERαKO) are peripherally insulin resistant, with impaired insulin signalling in skeletal muscle (despite normal GLUT4 levels), impaired fatty acid oxidation and accumulation of inflammatory lipid intermediates (Ribas *et al.* 2009). Estrogen replacement in ovariectomized mice shifts transcription patterns in skeletal muscle towards favouring fatty acid oxidation and rapidly activates AMPK (D'Eon *et al.* 2005). Whilst some reports suggests a negative effect on insulin sensitivity in the face of supraphysiological estradiol levels (Barros *et al.* 2008), the balance of evidence supports a salutary role for estrogens with respect to peripheral insulin sensitivity and offers a plausible explanation for the effects observed following aromatase inhibition. With hindsight, it would have been desirable to obtain skeletal muscle biopsies to determine anastrozole-induced changes in transcription and also to have considered assessment of intramyocellular lipid content.

Contrary to what was hypothesised, subcutaneous adipose tissue does not appear to significantly mediate the metabolic effects of aromatase inhibition in men. No effect upon lipolysis was detected through assessment of rate of appearance of glycerol. Furthermore, of 27 subcutaneous adipose mRNA transcript levels investigated, only 2 were found to be significantly different between treatment phases. The biological significance of slight reductions in perilipin 2 (more associated with lipid droplet formation in non-adipose cells) and estrogen receptor β is questionable. The proportion of subjects suitable (or willing) to have paired adipose biopsies was low (n

= 6) which reduced the power to detect differences, although there were no discernable trends in the remaining 25 genes. With the exception of leptin, no significant differences were observed in adipokines or pro-inflammatory cytokines, lending further support to a lack of meaningful effect upon the adipose compartment.

Ideally it would have been interesting to obtain visceral as well as subcutaneous adipose tissue, although this is not possible outwith the context of surgery. Needle biopsy of adipose tissue is associated with relative under sampling of the stroma-vascular fraction (Mutch *et al.* 2009), which is potentially relevant, as the pre-adipocyte fraction is particularly active with respect to sex steroid hormone metabolism. Assessment of estrogen sulfotransferase mRNA expression would be of value as another factor capable of significantly influencing tissue estrogen levels.

Anastrozole resulted in a significant reduction in total cholesterol, which comprised non-significant downward trends in both LDL and HDL cholesterol. This contrasts with a significant increase in LDL cholesterol, observed following 4 weeks of letrozole in younger men, although within this study no LDL cholesterol effect was observed in older men (Lapauw *et al.* 2009). A study of older men with 'mild hypogonadism' failed to demonstrate any significant difference in lipid profile following 12 weeks of anastrozole therapy (Dougherty *et al.* 2005). The most systematic assessment of changes in cholesterol associated with aromatase inhibition, involved 6-week administration of GnRH agonist, testosterone and testolactone (to determine the effects of estrogen deficiency); in young men this revealed aromatase inhibition was association with significant reductions in HDL cholesterol (primarily the HDL_2 fraction) (Bagatell *et al.* 1994). The less marked decline in HDL

cholesterol in the current study is likely to reflect less pronounced suppression of estradiol (in the absence of GnRH agonist).

Systolic blood pressure was significantly higher and heart rate significantly lower during the anastrozole phase of this study. The opposite phenotype is observed in aromatase knockout mice, namely increased heart rate and lower diastolic blood pressure (Head *et al.* 2004). Polymorphisms in *CYP19A1* have been associated with essential hypertension in a sex-specific manner (Shimodaira *et al.* 2008). The role of estrogen in influencing blood pressure is complex and evidence often conflicting. Most studies of HRT suggest a modest effect in reducing BP. Estrogens exert an influence upon endothelial function through a number of mechanisms (Yanes & Reckelhoff 2011), however a detailed exploration of the vascular effects of aromatase inhibition is beyond the scope of this thesis.

Summary

As hypothesised, in healthy men, aromatase inhibition resulted in decreased insulin sensitivity, primarily manifesting as reduced peripheral glucose disposal. No significant effects upon hepatic glucose output or lipolysis were observed. Anastrozole resulted in lower serum leptin levels, in the absence of any effect on leptin adipose mRNA expression, which potentially reflects either redistributed or reduced adiposity. Relatively modest changes in circulating sex steroid hormones may not accurately reflect differences in target tissue estrogen and androgen action.

Chapter 6

Conclusions

It is clear that sex steroid hormones exert an influence upon body composition and energy metabolism; multiple sources of evidence attest to this in animal models and in humans. Aromatase occupies a central role in determining estrogen levels in men and post-menopausal women both in the circulation and, perhaps more importantly, within target tissues. Models of aromatase deficiency are associated with adverse metabolic phenotypes yet, despite their widespread clinical use, little attention has focused on the potential metabolic consequences of aromatase inhibitors.

The study described in chapter 3 confirmed associations between sex steroids and measures of cardiometabolic risk in men. It also raised the possibility that, in men, availability of substrate androgen, rather than abundance of aromatase (using adiposity as a proxy), is of greater significance in determining plasma estrogen concentrations. However this study was subject to a number of important limitations, not least the inherent difficulty in establishing direction of association in a cross-sectional design. In addition, plasma steroid measurements were performed using immunometric assays, which are increasingly recognised to be unreliable at the lower end of expected concentration ranges (Huhtaniemi *et al.* 2012). This simple study raised questions regarding the extent to which adipose tissue and total body aromatase activity mediates circulating estradiol concentration in men. To address these questions, a study was designed where labelled androstenedione would be infused into subcutaneous adipose tissue, using microdialysis, with measurement of the resultant conversion to labelled estrone. Preliminary assessment of the feasibility of this approach (not reported in the thesis) identified a significant, ultimately insurmountable, problem with adsorption of sex steroid hormones to the plastic microdialysis tubing (Bruning *et al.* 1981). An alternative approach would be to

infuse stable isotope estrogen tracers systemically in order to measure whole body aromatisation; preparatory work was performed, not reported in the thesis, to establish the pharmacokinetics of stable estrogen tracers. However, challenges in the accurate LC-MS/MS measurement of estradiol at low picomolar concentrations thwarted progress in this regard to date. The flux of sex steroids through the aromatase pathway in men therefore remains unquantified. Another approach to deduce its significance, however, is to use enzyme inhibitors and this avenue proved fruitful, generating the results reported in chapters 4 and 5.

The study described in chapter 4 sought to assess the metabolic effects of aromatase inhibition in post-menopausal breast cancer patients, the main group exposed to this medication class in clinical practice. Aromatase inhibitors were associated with reduced lean mass, greater body fat percentage, significantly higher serum leptin and correspondingly greater leptin mRNA levels in subcutaneous adipose tissue. Aromatase inhibitor therapy was also associated with significantly greater insulin resistance, as determined by 'Matsuda-ISI'. Whilst cases and controls were matched as closely as possible, with no significant difference in age or BMI, case-control design is susceptible to confounding by unrecognised factors. Furthermore, although reported to correlate closely with the results of insulin infusion clamps (Matsuda *et al.* 1999), 'Matsuda-ISI' is not the gold standard method for assessing insulin sensitivity and does not inform the relative contributions of hepatic, adipose and peripheral insulin sensitivity.

The study described in chapter 5 represented an advance upon the earlier studies with respect to study design (randomised, double-blind, placebo-controlled crossover

study), assessment of insulin sensitivity (hyperinsulinaemic euglycaemic clamp with stable glucose and glycerol tracers) and measurement of sex steroid hormones (LC-MS/MS). Aromatase inhibition, in men, was associated with decreased insulin sensitivity; specifically, with reduced insulin-stimulated peripheral glucose disposal. Although sensitive modalities for detecting change in body composition were not employed, a fall in serum leptin is consistent with previously observed shifts from subcutaneous to visceral adiposity with aromatase inhibition in men (Finkelstein *et al*. 2013).

A significant deleterious effect upon insulin sensitivity was observed both in women (24.3% lower by 'Matsuda-ISI') and in men (23.7% decrease in peripheral glucose disposal during high dose clamp) following aromatase inhibitor therapy. This raises the possibility of an important pathogenic effect of aromatase inhibition in skeletal muscle, particularly as lean mass was lower in women treated with aromatase inhibitors. In support of this, estrogen deficiency impairs skeletal muscle glucose uptake (Campbell *et al.* 2002), reduces transcription of genes involved in muscle fatty acid oxidation (D'Eon *et al.* 2005) and reduces muscle AMPK signalling in rodent models (Rogers *et al.* 2009). The focus in this thesis had been on potential adipose tissue effects of aromatase inhibition but, in view of these findings, it would be interesting to assess effects upon intramyocellular lipid content in skeletal muscle and to determine whether significant changes in mRNA transcription are effected in muscle.

The effects of aromatase inhibition upon body composition may be sexually dimorphic. In women, higher serum leptin levels were observed in those receiving aromatase inhibitors whereas, in men, serum leptin fell following anastrozole administration. Detailed assessment of body composition was only performed in women, as the duration of the male study was not considered long enough to detect any significant differences. A low estrogen-high androgen state, as expected in aromatase inhibitor treated adipose tissue, would be predicted to favour android fat deposition (Elbers *et al.* 2003). However, body fat percentage was increased across both lower limbs and trunk. Future detailed assessment of changes in the visceral and subcutaneous adipose compartment would be of value. Previous investigations in men have shown aromatase inhibitors to preferentially increase visceral adiposity (Finkelstein *et al.* 2013), which is potentially consistent with the observed fall in serum leptin (Gottschling-Zeller *et al.* 1999).

The effect of aromatase inhibition upon mRNA transcript levels in subcutaneous adipose was minimal in men. In women, upregulation of genes associated with anti-adipogenic effects was observed (LKB1 and β-catenin) although leptin mRNA levels were greater, in keeping with higher serum levels. Overall, effects on transcription in adipose tissue were less than anticipated, based on previously described transcriptional effects of estrogens and androgens in adipose tissue.

Plasma sex steroid measurements were performed using gold standard LC-MS/MS methodology for the male aromatase inhibitor study reported in chapter 5, where all previous similar investigators have employed less reliable immunometric assays. The

degree of estradiol suppression by anastrozole was less than observed in previous similar studies and the absence of any effect upon circulating androgens was unanticipated (T'Sjoen *et al.* 2005); measurement of LH should help elucidate the reason for these findings. Measurement of SHBG is also planned, as a fall in SHBG may occur with estrogen suppression (Pasquali *et al,* 1997), leaving open the possibility of differences in calculated free testosterone. Ultimately, the most relevant sex steroid measurements in men and post-menopausal women are likely to be direct assessment of target tissue levels (*e.g.* adipose tissue or skeletal muscle), as circulating values are substantially lower and only passively reflect local tissue generation (Simpson *et al.* 2005). However, measurement of tissue sex steroid hormones is technically challenging, not to mention the difficulties in accessing the necessary tissue compartments. The possibility that changes in aromatase activity may be compensated for by changes in estrogen sulfotransferase (or other enzymes involved in estrogen metabolism) requires further assessment.

A potential confounder encountered in previous aromatase inhibitor studies is the suppressive effect of letrozole, although apparently not anastrozole (Buzdar *et al.* 2001), upon basal (Bisagni *et al.* 1996) and ACTH-stimulated cortisol (Bajetta *et al.* 1999). The fact that this effect appears to be durable over several months, by which point ACTH compensation would be expected, raises the possibility that the effect is mediated by changes in corticosteroid-binding globulin (CBG). Further elucidation of effects of aromatase inhibition upon the CBG concentration is planned. Previously reported effects of aromatase inhibition on the growth hormone axis, specifically a

fall in IGF-1 in younger men, also require further attention (Mauras *et al.* 2000; Lapauw *et al.* 2009).

Having established that aromatase inhibitors are likely to exert deleterious effects upon insulin sensitivity in post-menopausal women, it is important that larger studies are undertaken to confirm these findings. With over a million prescriptions issued annually for aromatase inhibitors in England alone (Prescribing and Primary Care Services, Health and Social Care Information Centre, 2013), there are potentially significant implications related to an increased risk of T2DM in this population.

Another interesting avenue for further investigation would be establishing whether increased aromatase activity is responsible for obesity related male hypogonadism, secondary to the expanded adipose pool of aromatase. Tracer studies assessing total body aromatase activity, as well as changes in estrogen generation across the adipose tissue arterio-venous gradient, would help resolves this contentious question. Aromatase inhibition employed in the treatment of hypogonadism in obese men, targeting normalisation of testosterone and estradiol, may not result in adverse metabolic consequences and is worthy of further investigation.

Addressing the original hypotheses advanced in this thesis:

1. Lower levels of circulating estrogens are associated with poorer metabolic health in both men and women: The results presented in chapter 3 support an association between plasma estrogens and metabolic health in men, albeit unavoidably confounded by androgen levels.

2. Aromatase inhibition is associated with deleterious effects upon body composition: The results presented in chapter 4 are consistent with an adverse effect upon body composition in aromatase inhibitor treated women.

3. Aromatase inhibition is associated with increased insulin resistance: Increased insulin resistance was observed in aromatase inhibitor treated women (chapter 4) and men (chapter 5).

4. Aromatase inhibition results in alterations in circulating adipokines and cytokines, through an effect on transcription in adipose tissue: A sexually dimorphic effect was observed with respect to leptin, which was higher in aromatase inhibitor treated women but fell during aromatase inhibitor therapy in men. Effects upon adipose transcription were limited, suggesting changes in another target tissue (*e.g.* skeletal muscle) may be of greater importance.

REFERENCES

Abate Nicola, Haffner Steven M., Garg Abhimanyu, Peshock Ronald M., Grundy Scott M.. Sex Steroid Hormones, Upper Body Obesity, and Insulin Resistance *J Clin Endocrinol Metab.* 2002;87:4522-4527.

Agarwal Veena R., Ashanullah Christy I., Simpson Evan R., Bulun Serdar E.. Alternatively Spliced Transcripts of the Aromatase Cytochrome P450 (CYP19) Gene in Adipose Tissue of Women *J Clin Endocrinol Metab.* 1997;82:70-74.

Ahima Rexford S., Stanley Takara L., Khor Victor K., Zanni Markella V., Grinspoon Steven K.. Estrogen Sulfotransferase Is Expressed in Subcutaneous Adipose Tissue of Obese Humans in Association with TNF-α and SOCS3 *The Journal of Clinical Endocrinology & Metabolism.* 2011;96:E1153-E1158.

Allan C A A., Strauss B J G J., Burger H G G., Forbes E A A., McLachlan R I I.. Testosterone Therapy Prevents Gain in Visceral Adipose Tissue and Loss of Skeletal Muscle in Non-obese Aging Men. *J Clin Endocrinol Metab.* 2007; 93(1): 139-46.

Amir Eitan, Seruga Bostjan, Niraula Saroj, Carlsson Lindsay, Ocaña Alberto. Toxicity of adjuvant endocrine therapy in postmenopausal breast cancer patients: a systematic review and meta-analysis. *Journal of the National Cancer Institute.* 2011;103:1299-1309.

Anderson Leah A., Mcternan Philip G., Barnett Anthony H., Kumar Sudhesh. The Effects of Androgens and Estrogens on Preadipocyte Proliferation in Human Adipose Tissue: Influence of Gender and Site *J Clin Endocrinol Metab.* 2001;86:5045-5051.

Anderson L. A., McTernan P. G., Harte A. L., Barnett A. H., Kumar S.. The regulation of HSL and LPL expression by DHT and flutamide in human subcutaneous adipose tissue. *Diabetes Obes Metab.* 2002;4:209-213.

Andersson Bjorn, Mattsson Lars-Ake, Hahn Lennart, et al. Estrogen Replacement Therapy Decreases Hyperandrogenicity and Improves Glucose Homeostasis and Plasma Lipids in Postmenopausal Women With Noninsulin-Dependent Diabetes Mellitus *J Clin Endocrinol Metab.* 1997;82:638-643.

Arabi A., Garnero P., Porcher R., Pelissier C., Benhamou C. L., Roux C.. Changes in body composition during post-menopausal hormone therapy: a 2 year prospective study *Hum. Reprod.*. 2003;18:1747-1752.
Arner P.. Human fat cell lipolysis: biochemistry, regulation and clinical role. *Best Pract Res Clin Endocrinol Metab.* 2005;19:471-482.

Armellini F., Zamboni M., Castelli S., et al. Interrelationships between intraabdominal fat and total serum testosterone levels in obese women. *Metabolism.* 1994;43:390-395.

Arnlöv J., Pencina M. J., Amin S., et al. Endogenous sex hormones and cardiovascular disease incidence in men. *Ann Intern Med.* 2006;145:176-184.

ATAC Trialists' Group. Comprehensive side-effect profile of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: long-term safety analysis of the ATAC trial *The Lancet Oncology.* 2006;7:633-643.

Bagatell C. J., Knopp R. H., Rivier J. E., Bremner W. J.. Physiological levels of estradiol stimulate plasma high density lipoprotein2 cholesterol levels in normal men. *The Journal of clinical endocrinology and metabolism.* 1994;78:855-861.

Baghaei Fariba, Rosmond Roland, Westberg Lars, et al. The CYP19 Gene and Associations with Androgens and Abdominal Obesity in Premenopausal Women *Obesity.* 2003;11:578-585.

Bajetta E., Zilembo N., Dowsett M., et al. Double-blind, randomised, multicentre endocrine trial comparing two letrozole doses, in postmenopausal breast cancer patients. *European journal of cancer (Oxford, England : 1990).* 1999;35:208-213.

Bajetta E., Martinetti A., Zilembo N., et al. Biological activity of anastrozole in postmenopausal patients with advanced breast cancer: effects on estrogens and bone metabolism *Annals of Oncology.* 2002;13:1059-1066.

Barros Rodrigo P., Machado Ubiratan F., Warner Margaret, Gustafsson Jan-Ake A.. Muscle GLUT4 regulation by estrogen receptors ERbeta and ERalpha. *Proceedings of the National Academy of Sciences of the United States of America.* 2006;103:1605-1608.

Barros Rodrigo Palazzo De Almeida P., Morani Andrea, Moriscot Anselmo, Machado Ubiratan Fabres F.. Insulin resistance of pregnancy involves estrogen-induced repression of muscle GLUT4. *Molecular and cellular endocrinology.* 2008;295:24-31.

Barros Rodrigo P., Gabbi Chiara, Morani Andrea, Warner Margaret, Gustafsson Jan-Ake A.. Participation of ERalpha and ERbeta in glucose homeostasis in skeletal muscle and white adipose tissue. *American journal of physiology. Endocrinology and metabolism.* 2009;297.

Basu Rita, Dalla Man Chiara, Campioni Marco, et al. Effects of Two Years of Testosterone Replacement on Insulin Secretion, Insulin Action, Glucose Effectiveness, Hepatic Insulin Clearance and Postprandial Glucose Turnover in Elderly Men. *Diabetes Care.* 2007; 30(8): 1972-8.

Bataille V., Perret B., Evans A., et al. Sex hormone-binding globulin is a major determinant of the lipid profile: the PRIME study. *Atherosclerosis.* 2005;179:369-373.

Behre Hermann M., Tammela Teuvo L., Arver Stefan, et al. A randomized, double-blind, placebo-controlled trial of testosterone gel on body composition and health-related quality-of-life in men with hypogonadal to low-normal levels of serum testosterone and symptoms of androgen deficiency over 6 months with 12 months open-label follow-up. *The aging male : the official journal of the International Society for the Study of the Aging Male.* 2012;15:198-207.

Bélanger C., Luu-The V., Dupont P., Tchernof A.. Adipose tissue intracrinology: potential importance of local androgen/estrogen metabolism in the regulation of adiposity. *Horm Metab Res.* 2002;34:737-745.

Bélanger C., Hould F. S., Lebel S., Biron S., Brochu G., Tchernof A.. Omental and subcutaneous adipose tissue steroid levels in obese men. *Steroids.* 2006;71:674-682.

Bellomo Giorgio, Sulas Maria Giovanna G., Mairate Elisabetta, Bardone Maria Beatrice B., Rolla Roberta. Hemolysis is a major cause of variability in insulin measurement during oral glucose tolerance test in children. *Clinical laboratory.* 2012;58:67-74.

Bhasin Shalender, Jasjua Guneet K., Pencina Michael, et al. Sex hormone-binding globulin, but not testosterone, is associated prospectively and independently with incident metabolic syndrome in men: the framingham heart study. *Diabetes care.* 2011;34:2464-2470.

Bhasin Shalender, Travison Thomas G., Storer Thomas W., et al. Effect of testosterone supplementation with and without a dual 5α-reductase inhibitor on fat-free mass in men with suppressed testosterone production: a randomized controlled trial. *JAMA : the journal of the American Medical Association.* 2012;307:931-939.

Bisagni G., Cocconi G., Scaglione F., Fraschini F., Ch , Trunet P. F.. Letrozole, a new oral non-steroidal aromastase inhibitor in treating postmenopausal patients with advanced breast cancer. A pilot study *Annals of Oncology.* 1996;7:99-102.

Blouin Karine, Richard Christian, Belanger Chantal, et al. Local Androgen Inactivation in Abdominal Visceral Adipose Tissue *J Clin Endocrinol Metab.* 2003;88:5944-5950.

Blouin Karine, Richard Christian, Brochu Gaétan, et al. Androgen inactivation and steroid-converting enzyme expression in abdominal adipose tissue in men. *The Journal of endocrinology.* 2006;191:637-649.

Bolduc C., Larose M., Yoshioka M., et al. Effects of dihydrotestosterone on adipose tissue measured by serial analysis of gene expression. *J Mol Endocrinol.* 2004;33:429-444.

Bolduc C., Yoshioka M., St-Amand J.. Transcriptomic characterization of the long-term dihydrotestosterone effects in adipose tissue. *Obesity (Silver Spring).* 2007;15:1107-1132.

Boulton K. L., Hudson D. U., Coppack S. W., Frayn K. N.. Steroid hormone interconversions in human adipose tissue in vivo. *Metabolism.* 1992;41:556-559.

Brandt P. A., Spiegelman D., Yaun S. S., et al. Pooled analysis of prospective cohort studies on height, weight, and breast cancer risk. *American journal of epidemiology.* 2000;152:514-527.

Brochu Martin, Tchernof Andre, Dionne Isabelle J., et al. What Are the Physical Characteristics Associated with a Normal Metabolic Profile Despite a High Level of Obesity in Postmenopausal Women? *J Clin Endocrinol Metab.* 2001;86:1020-1025.

Bruning P. F., Jonker K. M., Boerema-Baan A. W.. Adsorption of steroid hormones by plastic tubing. *J Steroid Biochem.* 1981;14:553-555.

Bryzgalova G., Gao H., Ahren B., et al. Evidence that oestrogen receptor-alpha plays an important role in the regulation of glucose homeostasis in mice: insulin sensitivity in the liver. *Diabetologia.* 2006;49:588-597.

Bryzgalova Galyna, Lundholm Lovisa, Portwood Neil, et al. Mechanisms of antidiabetogenic and body weight-lowering effects of estrogen in high-fat diet-fed mice. *American journal of physiology. Endocrinology and metabolism.* 2008;295:E904-912.

Bulun S. E., Simpson E. R.. Competitive reverse transcription-polymerase chain reaction analysis indicates that levels of aromatase cytochrome P450 transcripts in adipose tissue of buttocks, thighs, and abdomen of women increase with advancing age. *J Clin Endocrinol Metab.* 1994;78:428-432.

Bundred N. J.. The effects of aromatase inhibitors on lipids and thrombosis *British Journal of Cancer.* 2005;93:S23-S27.

Burke C. W., Anderson D. C.. Sex-hormone-binding globulin is an oestrogen amplifier. *Nature.* 1972;240:38-40.

Burnett-Bowie Sherri-Ann M., McKay Elizabeth A., Lee Hang, Leder Benjamin Z.. Effects of Aromatase Inhibition on Bone Mineral Density and Bone Turnover in Older Men with Low Testosterone Levels 2009;94:4785-4792.

Buzdar A., Howell A.. Advances in aromatase inhibition: clinical efficacy and tolerability in the treatment of breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2001;7:2620-2635.

Buzdar Aman U., Robertson John F. R., Eiermann Wolfgang, Nabholtz Jean-Marc. An overview of the pharmacology and pharmacokinetics of the newer generation aromatase inhibitors anastrozole, letrozole, and exemestane *Cancer.* 2002;95:2006-2016.

Campbell S. E., Febbraio M. A.. Effect of the ovarian hormones on GLUT4 expression and contraction-stimulated glucose uptake. *American journal of physiology. Endocrinology and metabolism.* 2002;282.

Cao Yuankui, Zhang Shaofen, Zou Shien, Xia Xian. The relationship between endogenous androgens and body fat distribution in early and late postmenopausal women. *PloS one.* 2013;8.

Carani Cesare, Qin Kenan, Simoni Manuela, et al. Effect of Testosterone and Estradiol in a Man with Aromatase Deficiency *N Engl J Med.* 1997;337:91-95.

Casabiell Xesús, Piñeiro Verónica, Peino Roberto, et al. Gender Differences in Both Spontaneous and Stimulated Leptin Secretion by Human Omental Adipose Tissue in Vitro : Dexamethasone and Estradiol Stimulate Leptin Release in Women, But Not in Men *The Journal of Clinical Endocrinology & Metabolism.* 1998;83:2149-2155.

Chen Zhao, Bassford Tamsen, Green Sylvan B., et al. Postmenopausal hormone therapy and body composition—a substudy of the estrogen plus progestin trial of the Women's Health Initiative *The American Journal of Clinical Nutrition.* 2005;82:651-656.

Choukem S. P., Gautier J. F.. How to measure hepatic insulin resistance? *Diabetes & Metabolism.* 2008;34:664-673.

Chu Micheline C., Cosper Pippa, Nakhuda Gary S., Lobo Rogerio A.. A comparison of oral and transdermal short-term estrogen therapy in postmenopausal women with metabolic syndrome. *Fertility and sterility.* 2006;86:1669-1675.

Clegg Deborah J., Brown Lynda M., Woods Stephen C., Benoit Stephen C.. Gonadal hormones determine sensitivity to central leptin and insulin. *Diabetes.* 2006;55:978-987.

Cleland W. H., Mendelson C. R., Simpson E. R.. Effects of aging and obesity on aromatase activity of human adipose cells. *J Clin Endocrinol Metab.* 1985;60:174-177.

Cnop Miriam, Landchild Melinda J., Vidal Josep, et al. The Concurrent Accumulation of Intra-Abdominal and Subcutaneous Fat Explains the Association Between Insulin Resistance and Plasma Leptin Concentrations : Distinct Metabolic Effects of Two Fat Compartments *Diabetes.* 2002;51:1005-1015.

Cooke Paul S., Naaz Afia. Role of Estrogens in Adipocyte Development and Function *Experimental Biology and Medicine.* 2004;229:1127-1135.

Corbould A. M., Bawden M. J., Lavranos T. C., Rodgers R. J., Judd S. J.. 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.* 2002;26:165-175.

Corbould A.. Chronic testosterone treatment induces selective insulin resistance in subcutaneous adipocytes of women. *J Endocrinol.* 2007;192:585-594.

Crave J. C., Lejeune H., Brébant C., Baret C., Pugeat M.. Differential effects of insulin and insulin-like growth factor I on the production of plasma steroid-binding globulins by human hepatoblastoma-derived (Hep G2) cells. *The Journal of clinical endocrinology and metabolism.* 1995;80:1283-1289.

Cristofanilli Massimo, Yamamura Yuko, Kau Shu-Wan W., et al. Thyroid hormone and breast carcinoma. Primary hypothyroidism is associated with a reduced incidence of primary breast carcinoma. *Cancer.* 2005;103:1122-1128.

Darimont C., Delansorne R., Paris J., Ailhaud G., Negrel R.. Influence of estrogenic status on the lipolytic activity of parametrial adipose tissue in vivo: an in situ microdialysis study. *Endocrinology.* 1997;138:1092-1096.

DeFronzo R. A., Tobin J. D., Andres R.. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *American Journal of Physiology - Endocrinology And Metabolism.* 1979;237:E214+.

D'Eon Tara M., Souza Sandra C., Aronovitz Mark, Obin Martin S., Fried Susan K., Greenberg Andrew S.. Estrogen Regulation of Adiposity and Fuel Partitioning: EVIDENCE OF GENOMIC AND NON-GENOMIC REGULATION OF LIPOGENIC AND OXIDATIVE PATHWAYS *J. Biol. Chem.*. 2005;280:35983-35991.

de Ronde W., Schouw Y. T., Muller M., et al. Associations of sex-hormone-binding globulin (SHBG) with non-SHBG-bound levels of testosterone and estradiol in independently living men. *The Journal of clinical endocrinology and metabolism.* 2005;90:157-162.

Dhindsa Sandeep, Miller Michael G., McWhirter Cecilia L., et al. Testosterone concentrations in diabetic and nondiabetic obese men. *Diabetes care.* 2010;33:1186-1192.

Dicker A., Rydén M., Näslund E., et al. Effect of testosterone on lipolysis in human pre-adipocytes from different fat depots. *Diabetologia.* 2004;47:420-428.

Dieudonne M. N., Pecquery R., Boumediene A., Leneveu M. C., Giudicelli Y.. Androgen receptors in human preadipocytes and adipocytes: regional specificities and regulation by sex steroids. *Am J Physiol.* 1998;274.

Dieudonne M. N., Pecquery R., Leneveu M. C., Giudicelli Y.. 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.* 2000;141:649-656.

Dieudonné M. N., Leneveu M. C., Giudicelli Y., Pecquery R.. Evidence for functional estrogen receptors alpha and beta in human adipose cells: regional specificities and regulation by estrogens. *American journal of physiology. Cell physiology.* 2004;286.

Dieudonne Marie-Noelle, Sammari Anes, Dos, Leneveu Marie-Christine, Giudicelli Yves, Pecquery Rene. Sex steroids and leptin regulate 11[beta]-hydroxysteroid dehydrogenase I and P450 aromatase expressions in human preadipocytes: Sex specificities *The Journal of Steroid Biochemistry and Molecular Biology.* 2006;99:189-196.

Ding Eric L., Song Yiqing, Malik Vasanti S., Liu Simin. Sex differences of endogenous sex hormones and risk of type 2 diabetes: a systematic review and meta-

analysis. *JAMA : the journal of the American Medical Association.* 2006;295:1288-1299.

Ding Eric L., Song Yiqing, Manson JoAnn E., et al. Sex Hormone–Binding Globulin and Risk of Type 2 Diabetes in Women and Men *N Engl J Med.* 2009;361:1152-1163.

Dougherty Ryan H., Rohrer Jacqueline L., Hayden Douglas, Rubin Stephen D., Leder Benjamin Z.. Effect of aromatase inhibition on lipids and inflammatory markers of cardiovascular disease in elderly men with low testosterone levels *Clinical Endocrinology.* 2005;62:228+.

Dowsett Mitch, Cuzick Jack, Ingle Jim, et al. Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. *Journal of clinical oncology.* 2010;28:509-518.

Dubey Raghvendra K., Oparil Suzanne, Imthurn Bruno, Jackson Edwin K.. Sex hormones and hypertension *Cardiovascular Research.* 2002;53:688-708.

Duren Dana L., Sherwood Richard J., Czerwinski Stefan A., et al. Body composition methods: comparisons and interpretation. *Journal of diabetes science and technology.* 2008;2:1139-1146.

Durnin J. V. G. A., Womersley J.. Body fat assessed from total body density and its estimation from skinfold thickness: measurements on 481 men and women aged from 16 to 72 Years *British Journal of Nutrition.* 1974;32:77-97.

Eick Geeta N., Thornton Joseph W.. Evolution of steroid receptors from an estrogen-sensitive ancestral receptor. *Molecular and cellular endocrinology.* 2011;334:31-38.

Elbers Jolanda M. H., Asscheman Henk, Seidell Jacob C., Frölich Marijke, Meinders A. Edo, Gooren Louis J. G.. Reversal of the Sex Difference in Serum Leptin Levels upon Cross-Sex Hormone Administration in Transsexuals *The Journal of Clinical Endocrinology & Metabolism.* 1997;82:3267-3270.

Elbers J. M., Asscheman H., Seidell J. C., Gooren L. J.. Effects of sex steroid hormones on regional fat depots as assessed by magnetic resonance imaging in transsexuals. *Am J Physiol.* 1999a;276.

Elbers J. M., Jong S., Teerlink T., Asscheman H., Seidell J. C., Gooren L. J.. 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.* 1999b;48:1371-1377.

Elbers J. M., Giltay E. J., Teerlink T., et al. Effects of sex steroids on components of the insulin resistance syndrome in transsexual subjects. *Clin Endocrinol (Oxf).* 2003;58:562-571.

Eriksson Anna L., Lorentzon Mattias, Vandenput Liesbeth, et al. Genetic Variations in Sex Steroid-Related Genes as Predictors of Serum Estrogen Levels in Men *J Clin Endocrinol Metab.* 2009;94:1033-1041.

Fan W., Yanase T., Nomura M., et al. Androgen receptor null male mice develop late-onset obesity caused by decreased energy expenditure and lipolytic activity but show normal insulin sensitivity with high adiponectin secretion. *Diabetes.* 2005;54:1000-1008.

Faqehi Abdullah MM, Cobice Diego F, Naredo Gregorio *et al.* Derivatisation of estrogens enhances specificity and sensitivity in human plasma by liquid chromatography tandem mass spectrometry. Submitted for publication 2014.

Ferrara Cynthia M., Lynch Nicole A., Nicklas Barbara J., Ryan Alice S., Berman Dora M.. Differences in Adipose Tissue Metabolism between Postmenopausal and Perimenopausal Women *J Clin Endocrinol Metab.* 2002;87:4166-4170.

Fields David A., Goran Michael I., McCrory Megan A.. Body-composition assessment via air-displacement plethysmography in adults and children: a review *The American Journal of Clinical Nutrition.* 2002;75:453-467.

Finkelstein Joel S., Lee Hang, Burnett-Bowie Sherri-Ann M., et al. Gonadal Steroids and Body Composition, Strength, and Sexual Function in Men *N Engl J Med.* 2013;369:1011-1022.

Fogelholm M., Marken Lichtenbelt W.. Comparison of body composition methods: a literature analysis. *European journal of clinical nutrition.* 1997;51:495-503.

Francini G., Petrioli R., Montagnani A., et al. Exemestane after tamoxifen as adjuvant hormonal therapy in postmenopausal women with breast cancer: effects on body composition and lipids. *British journal of cancer.* 2006;95:153-158.

Frias J. P., Macaraeg G. B., Ofrecio J., Yu J. G., Olefsky J. M., Kruszynska Y. T.. Decreased susceptibility to fatty acid-induced peripheral tissue insulin resistance in women. *Diabetes.* 2001;50:1344-1350.

Gabriely I., Ma X. H., Yang X. M., et al. Removal of visceral fat prevents insulin resistance and glucose intolerance of aging: an adipokine-mediated process? *Diabetes.* 2002;51:2951-2958.

Gambacciani M., Ciaponi M., Cappagli B., et al. Body weight, body fat distribution, and hormonal replacement therapy in early postmenopausal women. *The Journal of clinical endocrinology and metabolism.* 1997;82:414-417.

Gambineri A., Pelusi C., Genghini S., et al. Effect of flutamide and metformin administered alone or in combination in dieting obese women with polycystic ovary syndrome. *Clin Endocrinol (Oxf).* 2004;60:241-249.

Gambineri A., Patton L., Vaccina A., et al. Treatment with flutamide, metformin, and their combination added to a hypocaloric diet in overweight-obese women with polycystic ovary syndrome: a randomized, 12-month, placebo-controlled study. *J Clin Endocrinol Metab.* 2006;91:3970-3980.

Gao Qian, Horvath Tamas L.. Cross-talk between estrogen and leptin signaling in the hypothalamus. *American journal of physiology. Endocrinology and metabolism.* 2008;294.

Garrel D. R., Moussali R., De Oliveira A., Lesiège D., Larivière F.. RU 486 prevents the acute effects of cortisol on glucose and leucine metabolism. *The Journal of Clinical Endocrinology & Metabolism.* 1995;80:379-385.

Geisler Jürgen, Helle Hilgegunn, Ekse Dagfinn, et al. Letrozole is Superior to Anastrozole in Suppressing Breast Cancer Tissue and Plasma Estrogen Levels *Clinical Cancer Research.* 2008;14:6330-6335.

Genazzani A. R., Gambacciani M.. Effect of climacteric transition and hormone replacement therapy on body weight and body fat distribution. *Gynecol Endocrinol.* 2006;22:145-150.

Gennari Luigi, Masi Laura, Merlotti Daniela, et al. A polymorphic CYP19 TTTA repeat influences aromatase activity and estrogen levels in elderly men: effects on bone metabolism. *The Journal of clinical endocrinology and metabolism.* 2004;89:2803-2810.

Gianatti Emily J., Dupuis Philippe, Hoermann Rudolf, et al. Effect of Testosterone Treatment on Glucose Metabolism in men With Type 2 Diabetes: A Randomized Controlled Trial *Diabetes Care.* 2014:DC_132845+.

Giustarini E., Pinchera A., Fierabracci P., et al. Thyroid autoimmunity in patients with malignant and benign breast diseases before surgery. *European journal of endocrinology / European Federation of Endocrine Societies.* 2006;154:645-649.

Godsland I. F.. Oestrogens and insulin secretion. *Diabetologia.* 2005;48:2213-2220.

Gottschling-Zeller H., Birgel M., Scriba D., Blum W. F., Hauner H.. Depot-specific release of leptin from subcutaneous and omental adipocytes in suspension culture: effect of tumor necrosis factor-alpha and transforming growth factor-beta1. *European journal of endocrinology / European Federation of Endocrine Societies.* 1999;141:436-442.

Gray D. S., Bray G. A., Gemayel N., Kaplan K.. Effect of obesity on bioelectrical impedance. *The American journal of clinical nutrition.* 1989;50:255-260.

Greenfield M. S., Doberne L., Kraemer F., Tobey T., Reaven G.. Assessment of insulin resistance with the insulin suppression test and the euglycemic clamp. *Diabetes.* 1981;30:387-392.

Grey A. B., Stapleton J. P., Evans M. C., Reid I. R.. The effect of the anti-estrogen tamoxifen on cardiovascular risk factors in normal postmenopausal women. *The Journal of clinical endocrinology and metabolism.* 1995;80:3191-3195.

Gui Yaoting, Silha Josef V., Murphy Liam J.. Sexual Dimorphism and Regulation of Resistin, Adiponectin, and Leptin Expression in the Mouse *Obesity Research.* 2004;12:1481-1491.

Gupta Vandana, Bhasin Shalender, Guo Wen, et al. Effects of dihydrotestosterone on differentiation and proliferation of human mesenchymal stem cells and preadipocytes. *Molecular and cellular endocrinology.* 2008;296:32-40.

Gyllenborg J., Rasmussen S. L., Borch-Johnsen K., Heitmann B. L., Skakkebaek N. E., Juul A.. Cardiovascular risk factors in men: The role of gonadal steroids and sex hormone-binding globulin. *Metabolism: clinical and experimental.* 2001;50:882-888.

Haarbo J., Marslew U., Gotfredsen A., Christiansen C.. Postmenopausal hormone replacement therapy prevents central distribution of body fat after menopause. *Metabolism.* 1991;40:1323-1326.

Haffner S. M., Mykkanen L., Valdez R. A., Katz M. S.. Relationship of sex hormones to lipids and lipoproteins in nondiabetic men *J Clin Endocrinol Metab.* 1993;77:1610-1615.

Haffner S.M., Valdez, R.A., Mykkanen L et al. Decreased testosterone and dehydroepiandrosterone sulfate concentrations are associated with increased insulin and glucose concentrations in nondiabetic men *Metabolism.* 1994;43:599-603.

Haffner Steven M., Shaten Jessica, Stem Michael P., Smith George D., Kuller Lewis, Group Mrfit R.. Low Levels of Sex Hormone-binding Globulin and Testosterone Predict the Development of Non-Insulin-dependent Diabetes Mellitus in Men *Am. J. Epidemiol.*. 1996;143:889-897.

Hales C. N., Barker D. J., Clark P. M., et al. Fetal and infant growth and impaired glucose tolerance at age 64. *BMJ (Clinical research ed.).* 1991;303:1019-1022.

Hall J. M., McDonnell D. P.. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology.* 1999;140:5566-5578.

Hamilton E. J., Gianatti E., Strauss B. J., et al. Increase in visceral and subcutaneous abdominal fat in men with prostate cancer treated with androgen deprivation therapy *Clinical Endocrinology.* 2011;74:377-383.

Hammes Stephen R., Levin Ellis R.. Extranuclear Steroid Receptors: Nature and Actions *Endocrine Reviews.* 2007;28:726-741.

Hammes Annette, Andreassen Thomas K., Spoelgen Robert, et al. Role of Endocytosis in Cellular Uptake of Sex Steroids *Cell.* 2005;122:751-762.

Hamosh M., Hamosh P.. The effect of estrogen on the lipoprotein lipase activity of rat adipose tissue. *J Clin Invest.* 1975;55:1132-1135.

Hanchang Wanthanee, Semprasert Namoiy, Limjindaporn Thawornchai, Yenchitsomanus Pa-Thai T., Kooptiwut Suwattanee. Testosterone protects against glucotoxicity induced apoptosis of pancreatic β-cells (INS-1) and male mouse pancreatic islets. *Endocrinology.* 2013. 154(11): 4058-67.

Haring Robin, Volzke Henry, Felix Stephan B., et al. Prediction of Metabolic Syndrome by Low Serum Testosterone Levels in Men *Diabetes.* 2009;58:2027-2031.

Hayes Frances J., Seminara Stephanie B., DeCruz Suzzunne, Boepple Paul A., Crowley William F.. Aromatase Inhibition in the Human Male Reveals a Hypothalamic Site of Estrogen Feedback *Journal of Clinical Endocrinology & Metabolism.* 2000;85:3027-3035.

Head Geoffrey A., Obeyesekere Varuni R., Jones Margaret E., Simpson Evan R., Krozowski Zygmunt S.. Aromatase-Deficient (ArKO) Mice Have Reduced Blood Pressure and Baroreflex Sensitivity *Endocrinology.* 2004;145:4286-4291.

Health and Social Care Information Cenre, Government Statistical Service. Prescriptions Dispensed in the Community: England 2002 – 2012. 2012.

Heine P. A., Taylor J. A., Iwamoto G. A., Lubahn D. B., Cooke P. S.. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proceedings of the National Academy of Sciences of the United States of America.* 2000;97:12729-12734.

Herrmann Burkhard Lorenz L., Saller Bernhard, Janssen Onno Eilard E., et al. Impact of estrogen replacement therapy in a male with congenital aromatase deficiency caused by a novel mutation in the CYP19 gene. *The Journal of clinical endocrinology and metabolism.* 2002;87:5476-5484.

Heufelder Armin E., Saad Farid, Bunck Mathijs C., Gooren Louis. Fifty-two-Week Treatment With Diet and Exercise Plus Transdermal Testosterone Reverses the Metabolic Syndrome and Improves Glycemic Control in Men With Newly Diagnosed Type 2 Diabetes and Subnormal Plasma Testosterone *J Androl.* 2009;30:726-733.

Hewitt Kylie N., Pratis Kyriakos, Jones Margaret E., Simpson Evan R.. Estrogen Replacement Reverses the Hepatic Steatosis Phenotype in the Male Aromatase Knockout Mouse *Endocrinology.* 2004;145:1842-1848.

Holmäng A., Björntorp P.. The effects of testosterone on insulin sensitivity in male rats. *Acta physiologica Scandinavica.* 1992;146:505-510.

Homma Hiroaki, Kurachi Hirohisa, Nishio Yukihiro, et al. Estrogen Suppresses Transcription of Lipoprotein Lipase Gene. EXISTENCE OF A UNIQUE

ESTROGEN RESPONSE ELEMENT ON THE LIPOPROTEIN LIPASE PROMOTER *J. Biol. Chem.*. 2000;275:11404-11411.

Huang Seng-Wong, Seow Kok-Min, Ho Low-Tone, et al. Resistin mRNA levels are downregulated by estrogen in vivo and in vitro *FEBS Letters.* 2005;579:449-454.

Huhtaniemi Ilpo T., Tajar Abdelouahid, Lee David M., et al. Comparison of serum testosterone and estradiol measurements in 3174 European men using platform immunoassay and mass spectrometry; relevance for the diagnostics in aging men. *European journal of endocrinology / European Federation of Endocrine Societies.* 2012;166:983-991.

Ihunnah Chibueze A., Wada Taira, Philips Brian J., et al. Estrogen Sulfotransferase/SULT1E1 Promotes Human Adipogenesis *Molecular and Cellular Biology.* 2014;34:1682-1694.

Iverius P. H., Brunzell J. D.. Relationship between lipoprotein lipase activity and plasma sex steroid level in obese women. *J Clin Invest.* 1988;82:1106-1112.

Jasuja Guneet Kaur K., Travison Thomas G., Davda Maithili, et al. Circulating estrone levels are associated prospectively with diabetes risk in men of the framingham heart study. *Diabetes care.* 2013;36:2591-2596.

Jones M. E., Thorburn A. W., Britt K. L., et al. Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity. *Proc Natl Acad Sci U S A.* 2000;97:12735-12740.

Jones M. E., Thorburn A. W., Britt K. L., et al. Aromatase-deficient (ArKO) mice accumulate excess adipose tissue. *J Steroid Biochem Mol Biol.* 2001;79:3-9.

Jones Margaret E., Boon Wah Chin C., Proietto Joseph, Simpson Evan R.. Of mice and men: the evolving phenotype of aromatase deficiency. *Trends in endocrinology and metabolism: TEM.* 2006;17:55-64.

Jones T. Hugh, Arver Stefan, Behre Hermann M., et al. Testosterone Replacement in Hypogonadal Men With Type 2 Diabetes and/or Metabolic Syndrome (the TIMES2 Study) *Diabetes Care.* 2011;34:828-837.

Johnson Richard J., Perez-Pozo Santos E., Sautin Yuri Y., et al. Hypothesis: Could Excessive Fructose Intake and Uric Acid Cause Type 2 Diabetes? *Endocrine Reviews.* 2009;30:96-116.

Kahn S. M., Hryb D. J., Nakhla A. M., Romas N. A., Rosner W.. Sex hormone-binding globulin is synthesized in target cells *J Endocrinol.* 2002;175:113-120.

Kalme T., Seppälä M., Qiao Q., et al. Sex hormone-binding globulin and insulin-like growth factor-binding protein-1 as indicators of metabolic syndrome, cardiovascular risk, and mortality in elderly men. *J Clin Endocrinol Metab.* 2005;90:1550-1556.

Kalyani Rita Rastogi R., Franco Manuel, Dobs Adrian S., et al. The association of endogenous sex hormones, adiposity, and insulin resistance with incident diabetes in postmenopausal women. *The Journal of clinical endocrinology and metabolism.* 2009;94:4127-4135.

Kanaya Alka M., Herrington David, Vittinghoff Eric, et al. Glycemic effects of postmenopausal hormone therapy: the Heart and Estrogen/progestin Replacement Study. A randomized, double-blind, placebo-controlled trial. *Annals of internal medicine.* 2003;138:1-9.

Kapoor D., Clarke S., Stanworth R., Channer K. S., Jones T. H.. The effect of testosterone replacement therapy on adipocytokines and C-reactive protein in hypogonadal men with type 2 diabetes *European Journal of Endocrinology.* 2007;156:595-602.

Katsuki A., Sumida Y., Murashima S., et al. Acute and chronic regulation of serum sex hormone-binding globulin levels by plasma insulin concentrations in male noninsulin-dependent diabetes mellitus patients *J Clin Endocrinol Metab.* 1996;81:2515-2519.

Katz A., Nambi S. S., Mather K., et al. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *The Journal of clinical endocrinology and metabolism.* 2000;85:2402-2410.

Keating Nancy L., O'Malley A. James, Smith Matthew R.. Diabetes and Cardiovascular Disease During Androgen Deprivation Therapy for Prostate Cancer *Journal of Clinical Oncology.* 2006;24:4448-4456.

Kelly Daniel M., Jones T. Hugh. Testosterone: a metabolic hormone in health and disease. *The Journal of endocrinology.* 2013;217.

Khor Victor K., Dhir Ravindra, Yin Xiaoyan, Ahima Rexford S., Song Wen-Chao. Estrogen sulfotransferase regulates body fat and glucose homeostasis in female mice *American Journal of Physiology - Endocrinology and Metabolism.* 2010;299:E657-E664.

Killinger D. W., Perel E., Danilescu D., Kharlip L., Lindsay W. R.. Influence of adipose tissue distribution on the biological activity of androgens. *Ann N Y Acad Sci.* 1990;595:199-211.

Kim Catherine, Edelstein Sharon L., Crandall Jill P., et al. Menopause and risk of diabetes in the Diabetes Prevention Program. *Menopause (New York, N.Y.).* 2011;18:857-868.

Kirchengast Sylvia, Huber Johannes. Body composition characteristics and fat distribution patterns in young infertile women. *Fertility and sterility.* 2004;81:539-544.

Kley H. K., Deselaers T., Peerenboom H., Krüskemper H. L.. Enhanced conversion of androstenedione to estrogens in obese males. *J Clin Endocrinol Metab.* 1980;51:1128-1132.

Kley H. K., Schlaghecke R., Krüskemper H. L.. [Stability of steroids in plasma over a 10-year period] *Journal of clinical chemistry and clinical biochemistry. Zeitschrift für klinische Chemie und klinische Biochemie.* 1985;23:875-878.

Kormas N., Diamond T., O'Sullivan A., Smerdely P.. Body mass and body composition after total thyroidectomy for benign goiters. *Thyroid.* 1998;8:773-776.

Kritz-Silverstein D., Barrett-Connor E.. Long-term postmenopausal hormone use, obesity, and fat distribution in older women. *JAMA.* 1996;275:46-49.

Kuk Jennifer L., Lee Sojung, Heymsfield Steven B., Ross Robert. Waist circumference and abdominal adipose tissue distribution: influence of age and sex *Am J Clin Nutr.* 2005;81:1330-1334.

Kouzmenko Alexander P., Takeyama Ken-ichi, Ito Saya, et al. Wnt/β-Catenin and Estrogen Signaling Converge in Vivo *Journal of Biological Chemistry.* 2004;279:40255-40258.

Kupelian Varant, Page Stephanie T., Araujo Andre B., Travison Thomas G., Bremner William J., Mckinlay John B.. Low Sex Hormone-Binding Globulin, Total Testosterone, and Symptomatic Androgen Deficiency Are Associated with Development of the Metabolic Syndrome in Nonobese Men *J Clin Endocrinol Metab.* 2006;91:843-850.

Laaksonen David E., Niskanen Leo, Punnonen Kari, et al. Testosterone and Sex Hormone-Binding Globulin Predict the Metabolic Syndrome and Diabetes in Middle-Aged Men *Diabetes Care.* 2004;27:1036-1041.

Laaksonen David E., Niskanen Leo, Punnonen Kari, et al. The Metabolic Syndrome and Smoking in Relation to Hypogonadism in Middle-Aged Men: A Prospective Cohort Study *J Clin Endocrinol Metab.* 2005;90:712-719.

Lac G., Marquet P., Chassain A. P., Galen F. X.. Dexamethasone in resting and exercising men. II. Effects on adrenocortical hormones *J Appl Physiol.* 1999;87:183-188.

Lafontan M., Berlan M.. Do regional differences in adipocyte biology provide new pathophysiological insights? *Trends Pharmacol Sci.* 2003;24:276-283.

Lanfranco Fabio, Zitzmann Michael, Simoni Manuela, Nieschlag Eberhard. Serum adiponectin levels in hypogonadal males: influence of testosterone replacement therapy. *Clinical endocrinology.* 2004;60:500-507.

Lapauw B., T'Sjoen G., Mahmoud A., Kaufman J. M., Ruige J. B.. Short-term aromatase inhibition: effects on glucose metabolism and serum leptin levels in young and elderly men *European Journal of Endocrinology.* 2009;160:397-402.

Lapauw Bruno, Ouwens Margriet, Hart Leen M., et al. Sex Steroids Affect Triglyceride Handling, Glucose-Dependent Insulinotropic Polypeptide, and Insulin Sensitivity *Diabetes Care.* 2010;33:1831-1833.

Lapidus L., Lindstedt G., Lundberg P. A., Bengtsson C., Gredmark T.. Concentrations of sex-hormone binding globulin and corticosteroid binding globulin in serum in relation to cardiovascular risk factors and to 12-year incidence of cardiovascular disease and overall mortality in postmenopausal women. *Clinical chemistry.* 1986;32:146-152.

Larionov A. A., Vasyliev D. A., Mason J. I., Howie A. F., Berstein L. M., Miller W. R.. Aromatase in skeletal muscle. *The Journal of steroid biochemistry and molecular biology.* 2003;84:485-492.

Lemieux S., Després J. P., Moorjani S., et al. Are gender differences in cardiovascular disease risk factors explained by the level of visceral adipose tissue? *Diabetologia.* 1994;37:757-764.

Linder Kristina, Arner Peter, Flores-Morales Amilcar, Tollet-Egnell Petra, Norstedt Gunnar. Differentially expressed genes in visceral or subcutaneous adipose tissue of obese men and women. *Journal of lipid research.* 2004;45:148-154.

Liu Suhuan, Le May Cedric, Wong Winifred P., et al. Importance of extranuclear estrogen receptor-alpha and membrane G protein-coupled estrogen receptor in pancreatic islet survival. *Diabetes.* 2009;58:2292-2302.

Lo Joan C., Zhao Xinhua, Scuteri Angelo, Brockwell Sarah, Sowers MaryFran R.. The association of genetic polymorphisms in sex hormone biosynthesis and action with insulin sensitivity and diabetes mellitus in women at midlife. *The American journal of medicine.* 2006;119.

Longcope C., Pratt J. H., Schneider S. H., Fineberg S. E.. Aromatization of androgens by muscle and adipose tissue in vivo. *The Journal of clinical endocrinology and metabolism.* 1978;46:146-152.

Longcope C., Baker R., Johnston C. C.. Androgen and estrogen metabolism: relationship to obesity. *Metabolism.* 1986;35:235-237.

Lovejoy J. C., Bray G. A., Bourgeois M. O., et al. Exogenous androgens influence body composition and regional body fat distribution in obese postmenopausal women-a clinical research center study. *The Journal of clinical endocrinology and metabolism.* 1996;81:2198-2203.

Loves Sandra, Ruinemans-Koerts Janneke, Boer Hans. Letrozole once a week normalizes serum testosterone in obesity-related male hypogonadism *Eur J Endocrinol.* 2008;158:741-747.

Machinal F., Dieudonne M. N., Leneveu M. C., Pecquery R., Giudicelli Y.. In vivo and in vitro ob gene expression and leptin secretion in rat adipocytes: evidence for a

regional specific regulation by sex steroid hormones. *Endocrinology.* 1999;140:1567-1574.

Maliqueo Manuel, Sun Miao, Johansson Julia, et al. Continuous Administration of a P450 Aromatase Inhibitor Induces Polycystic Ovary Syndrome with a Metabolic and Endocrine Phenotype in Female Rats at Adult Age *Endocrinology.* 2013;154:434-445.

Margolis K. L., Bonds D. E., Rodabough R. J., et al. Effect of oestrogen plus progestin on the incidence of diabetes in postmenopausal women: results from the Women's Health Initiative Hormone Trial. *Diabetologia.* 2004;47:1175-1187.

Mårin P., Holmäng S., Jönsson L., et al. The effects of testosterone treatment on body composition and metabolism in middle-aged obese men. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity.* 1992;16:991-997.

Mather Kieren J., Hunt Evay A., Steinberg Helmut O., et al. Repeatability Characteristics of Simple Indices of Insulin Resistance: Implications for Research Applications *J Clin Endocrinol Metab.* 2001;86:5457-5464.

Matsuda M., DeFronzo R. A.. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care.* 1999;22:1462-1470.

Matthews D. R., Hosker J. P., Rudenski A. S., Naylor B. A., Treacher D. F., Turner R. C.. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia.* 1985;28:412-419.

Mauras N., O'Brien K. O., Klein K. O., Hayes V.. Estrogen suppression in males: metabolic effects. *The Journal of clinical endocrinology and metabolism.* 2000;85:2370-2377.

Mauriege P., Imbeault P., Prud'homme D., Tremblay A., Nadeau A., Despres J. P.. Subcutaneous Adipose Tissue Metabolism at Menopause: Importance of Body Fatness and Regional Fat Distribution *J Clin Endocrinol Metab.* 2000;85:2446-2454.

Mauvais-Jarvis Franck, Clegg Deborah J., Hevener Andrea L.. The role of estrogens in control of energy balance and glucose homeostasis. *Endocrine reviews.* 2013;34:309-338.

McInnes Kerry J., Corbould Anne, Simpson Evan R., Jones Margaret E.. Regulation of adenosine 5',monophosphate-activated protein kinase and lipogenesis by androgens contributes to visceral obesity in an estrogen-deficient state. *Endocrinology.* 2006;147:5907-5913.

McInnes Kerry J., Smith Lee B., Hunger Nicole I., Saunders Philippa T., Andrew Ruth, Walker Brian R.. Deletion of the androgen receptor in adipose tissue in male mice elevates retinol binding protein 4 and reveals independent effects on visceral fat mass and on glucose homeostasis. *Diabetes.* 2012;61:1072-1081.

Mcternan Philip G., Anderson Leah A., Anwar Aresh J., et al. Glucocorticoid Regulation of P450 Aromatase Activity in Human Adipose Tissue: Gender and Site Differences *J Clin Endocrinol Metab.* 2002;87:1327-1336.

Mendel C. M.. The free hormone hypothesis: a physiologically based mathematical model. *Endocrine reviews.* 1989;10:232-274.

Miyajima Mari, Kusuhara Hiroyuki, Takahashi Kayo, et al. Investigation of the effect of active efflux at the blood–brain barrier on the distribution of nonsteroidal aromatase inhibitors in the central nervous system *J. Pharm. Sci.*. 2013;102:3309-3319.

Mikkola T. S., Clarkson T. B.. Estrogen replacement therapy, atherosclerosis, and vascular function. *Cardiovasc Res.* 2002;53:605-619.

Miller K. K., Biller B. M. K., Schaub A., et al. Effects of Testosterone Therapy on Cardiovascular Risk Markers in Androgen-Deficient Women with Hypopituitarism *J Clin Endocrinol Metab.* 2007;92:2474-2479.

Misso Marie L., Murata Yoko, Boon Wah C., Jones Margaret E., Britt Kara L., Simpson Evan R.. Cellular and Molecular Characterization of the Adipose Phenotype of the Aromatase-Deficient Mouse *Endocrinology.* 2003;144:1474-1480.

Möhlig Matthias, Arafat Ayman M., Osterhoff Martin A., et al. Androgen receptor CAG repeat length polymorphism modifies the impact of testosterone on insulin sensitivity in men. *European journal of endocrinology / European Federation of Endocrine Societies.* 2011;164:1013-1018.

Montague C. T., Prins J. B., Sanders L., et al. Depot-related gene expression in human subcutaneous and omental adipocytes. *Diabetes.* 1998;47:1384-1391.

Moran Antoinette, Jacobs David R., Steinberger Julia, et al. Changes in insulin resistance and cardiovascular risk during adolescence: establishment of differential risk in males and females. *Circulation.* 2008;117:2361-2368.

Morimoto S., Fernandez-Mejia C., Romero-Navarro G., Morales-Peza N., Díaz-Sánchez V.. Testosterone effect on insulin content, messenger ribonucleic acid levels, promoter activity, and secretion in the rat. *Endocrinology.* 2001;142:1442-1447.

Morishima A., Grumbach M. M., Simpson E. R., Fisher C., Qin K.. Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. *The Journal of clinical endocrinology and metabolism.* 1995;80:3689-3698.

Moser Kath, Patnick Julietta, Beral Valerie. Inequalities in reported use of breast and cervical screening in Great Britain: analysis of cross sectional survey data. *BMJ (Clinical research ed.).* 2009;338.

Mosteller R. D.. Simplified calculation of body-surface area. *The New England journal of medicine.* 1987;317.

Movérare-Skrtic S., Venken K., Andersson N., et al. Dihydrotestosterone treatment results in obesity and altered lipid metabolism in orchidectomized mice. *Obesity (Silver Spring).* 2006;14:662-672.

Muller Majon, Grobbee Diederick E., Tonkelaar Isolde, Lamberts Steven W., Schouw Yvonne T.. Endogenous Sex Hormones and Metabolic Syndrome in Aging Men *J Clin Endocrinol Metab.* 2005;90:2618-2623.

Muniyappa Ranganath, Lee Sihoon, Chen Hui, Quon Michael J.. Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage. *American journal of physiology. Endocrinology and metabolism.* 2008;294:E15-E26.

Mutch David M., Tordjman Joan, Pelloux Véronique, et al. Needle and surgical biopsy techniques differentially affect adipose tissue gene expression profiles *The American Journal of Clinical Nutrition.* 2009;89:51-57.

Muthusamy Thirupathi, Murugesan Palaniappan, Balasubramanian Karundevi. Sex steroids deficiency impairs glucose transporter 4 expression and its translocation through defective Akt phosphorylation in target tissues of adult male rat. *Metabolism: clinical and experimental.* 2009;58:1581-1592.

Muthusamy Thirupathi, Murugesan Palaniappan, Srinivasan Chinnapaiyan, Balasubramanian Karundevi. Sex steroids influence glucose oxidation through modulation of insulin receptor expression and IRS-1 serine phosphorylation in target tissues of adult male rat. *Molecular and cellular biochemistry.* 2011;352:35-45.

Naaz A., Zakroczymski M., Heine P., et al. Effect of ovariectomy on adipose tissue of mice in the absence of estrogen receptor alpha (ERalpha): a potential role for estrogen receptor beta (ERbeta). *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et métabolisme.* 2002;34:758-763.

Nair K. S., Rizza R. A., O'Brien P., et al. DHEA in elderly women and DHEA or testosterone in elderly men. *N Engl J Med.* 2006;355:1647-1659.

Nakagawa T., Hu H., Zharikov S., et al. A causal role for uric acid in fructose-induced metabolic syndrome. *American journal of physiology. Renal physiology.* 2006;290.

Nantermet Pascale, Harada Shun-Ichi I., Liu Yuan, et al. Gene Expression Analyses in Cynomolgus Monkeys Provides Mechanistic Insight into High Density Lipoprotein-Cholesterol Reduction by Androgens in Primates. *Endocrinology.* 2008.

Nguyen M. C., Stewart R. B., Banerji M. A., Gordon D. H., Kral J. G.. Relationships between tamoxifen use, liver fat and body fat distribution in women with breast cancer. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity.* 2001;25:296-298.

Nielsen S., Guo Z., Johnson C. M., Hensrud D. D., Jensen M. D.. Splanchnic lipolysis in human obesity. *J Clin Invest.* 2004;113:1582-1588.

Nielsen Torben L., Hagen Claus, Wraae Kristian, et al. Visceral and Subcutaneous Adipose Tissue Assessed by Magnetic Resonance Imaging in Relation to Circulating Androgens, Sex Hormone-Binding Globulin, and Luteinizing Hormone in Young Men *Journal of Clinical Endocrinology & Metabolism.* 2007;92:2696-2705.

Nigro Patrizia, Satoh Kimio, O'Dell Michael R., et al. Cyclophilin A is an inflammatory mediator that promotes atherosclerosis in apolipoprotein E-deficient mice. *The Journal of experimental medicine.* 2011;208:53-66.

Nohara Kazunari, Zhang Yan, Waraich Rizwana S., et al. Early-Life Exposure to Testosterone Programs the Hypothalamic Melanocortin System *Endocrinology.* 2011;152:1661-1669.

Oh Jee-Young, Barrett-Connor Elizabeth, Wedick Nicole M., Wingard Deborah L.. Endogenous Sex Hormones and the Development of Type 2 Diabetes in Older Men and Women: the Rancho Bernardo Study *Diabetes Care.* 2002;25:55-60.

Olofsson Louise E., Pierce Andrew A., Xu Allison W.. Functional requirement of AgRP and NPY neurons in ovarian cycle-dependent regulation of food intake. *Proceedings of the National Academy of Sciences of the United States of America.* 2009;106:15932-15937.

Page Stephanie T., Amory John K., Bowman F. DuBois, et al. Exogenous Testosterone (T) Alone or with Finasteride Increases Physical Performance, Grip Strength, and Lean Body Mass in Older Men with Low Serum T *Journal of Clinical Endocrinology & Metabolism.* 2005;90:1502-1510.

Palin S. L., McTernan P. G., Anderson L. A., Sturdee D. W., Barnett A. H., Kumar S.. 17Beta-estradiol and anti-estrogen ICI:compound 182,780 regulate expression of lipoprotein lipase and hormone-sensitive lipase in isolated subcutaneous abdominal adipocytes. *Metabolism.* 2003;52:383-388.

Palomar-Morales Martín, Morimoto Sumiko, Mendoza-Rodríguez Carmen Adriana A., Cerbón Marco A.. The protective effect of testosterone on streptozotocin-induced apoptosis in beta cells is sex specific. *Pancreas.* 2010;39:193-200.

Pasquali R., Casimirri F., De Iasio R., et al. Insulin regulates testosterone and sex hormone-binding globulin concentrations in adult normal weight and obese men *J Clin Endocrinol Metab.* 1995;80:654-658.

Pasquali R., Vicennati V., Bertazzo D., et al. Determinants of sex hormone-binding globulin blood concentrations in premenopausal and postmenopausal women with different estrogen status. Virgilio-Menopause-Health Group. *Metabolism: clinical and experimental.* 1997;46:5-9.

Pasqualini J. R., Chetrite G., Blacker C., et al. Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients. *The Journal of clinical endocrinology and metabolism.* 1996;81:1460-1464.

Payne Anita H., Hales Dale B.. Overview of Steroidogenic Enzymes in the Pathway from Cholesterol to Active Steroid Hormones *Endocr Rev.* 2004;25:947-970.

Pedersen S. B., Bruun J. M., Hube F., Kristensen K., Hauner H., Richelsen B.. Demonstration of estrogen receptor subtypes alpha and beta in human adipose tissue: influences of adipose cell differentiation and fat depot localization. *Mol Cell Endocrinol.* 2001;182:27-37.

Pedersen S. B., Bruun J. M., Kristensen K., Richelsen B.. Regulation of UCP1, UCP2, and UCP3 mRNA expression in brown adipose tissue, white adipose tissue, and skeletal muscle in rats by estrogen. *Biochemical and biophysical research communications.* 2001;288:191-197.

Pedersen Steen B., Kristensen Kurt, Hermann Pernille A., Katzenellenbogen John A., Richelsen Bjørn. Estrogen controls lipolysis by up-regulating alpha2A-adrenergic receptors directly in human adipose tissue through the estrogen receptor alpha. Implications for the female fat distribution. *The Journal of clinical endocrinology and metabolism.* 2004;89:1869-1878.

Perry John R. B., Weedon Michael N., Langenberg Claudia, et al. Genetic evidence that raised sex hormone binding globulin (SHBG) levels reduce the risk of type 2 diabetes *Human Molecular Genetics.* 2010;19:535-544.

Peter Inga, Shearman Amanda M., Zucker Deborah R., et al. Variation in estrogen-related genes and cross-sectional and longitudinal blood pressure in the Framingham Heart Study. *Journal of hypertension.* 2005;23:2193-2200.

Pfeilschifter Johannes, Köditz Roland, Pfohl Martin, Schatz Helmut. Changes in proinflammatory cytokine activity after menopause. *Endocrine reviews.* 2002;23:90-119.

Pitteloud Nelly, Mootha Vamsi K., Dwyer Andrew A., et al. Relationship Between Testosterone Levels, Insulin Sensitivity, and Mitochondrial Function in Men *Diabetes Care.* 2005;28:1636-1642.

Plymate S. R., Leonard J. M., Paulsen C. A., Fariss B. L., Karpas A. E.. Sex hormone-binding globulin changes with androgen replacement. *The Journal of clinical endocrinology and metabolism.* 1983;57:645-648.

Plymate S. R., Matej L. A., Jones R. E., Friedl K. E.. Inhibition of sex hormone-binding globulin production in the human hepatoma (Hep G2) cell line by insulin and prolactin. *The Journal of clinical endocrinology and metabolism.* 1988;67:460-464.

Plymate S.. Regulation of sex hormone-binding globulin production by growth factors, *Metabolism.* 1990;39:967-970.

Poehlman Eric T.. Menopause, energy expenditure, and body composition. *Acta obstetricia et gynecologica Scandinavica.* 2002;81:603-611.

Pritchard J. E., Nowson C. A., Strauss B. J., Carlson J. S., Kaymakci B., Wark J. D.. Evaluation of dual energy X-ray absorptiometry as a method of measurement of body fat. *European journal of clinical nutrition.* 1993;47:216-228.

Pritchard Janet, Despres Jean-Pierre, Gagnon Jacques, et al. Plasma Adrenal, Gonadal, and Conjugated Steroids before and after Long Term Overfeeding in Identical Twins *J Clin Endocrinol Metab.* 1998;83:3277-3284.

Quinkler Marcus, Sinha Binayak, Tomlinson Jeremy W., Bujalska Iwona J., Stewart Paul M., Arlt Wiebke. Androgen generation in adipose tissue in women with simple obesity - a site-specific role for 17beta-hydroxysteroid dehydrogenase type 5 *J Endocrinol.* 2004;183:331-342.

Ramachandran Surya, Venugopal Anila, Sathisha K., et al. Proteomic profiling of high glucose primed monocytes identifies cyclophilin A as a potential secretory marker of inflammation in type 2 diabetes. *Proteomics.* 2012;12:2808-2821.

Raven Garrett, Jong Frank H., Kaufman Jean-Marc, Ronde Willem. In Men, Peripheral Estradiol Levels Directly Reflect the Action of Estrogens at the Hypothalamo-Pituitary Level to Inhibit Gonadotropin Secretion *Journal of Clinical Endocrinology & Metabolism.* 2006;91:3324-3328.

Rebuffé-Scrive M., Mårin P., Björntorp P.. Effect of testosterone on abdominal adipose tissue in men. *Int J Obes.* 1991;15:791-795.

Reynolds Rebecca M., Walker Brian R., Syddall Holly E., et al. Altered Control of Cortisol Secretion in Adult Men with Low Birth Weight and Cardiovascular Risk Factors *J Clin Endocrinol Metab.* 2001;86:245-250.

Ribas Vicent, Nguyen M. T. Audrey, Henstridge Darren C., et al. Impaired oxidative metabolism and inflammation are associated with insulin resistance in ERα-deficient mice *American Journal of Physiology - Endocrinology and Metabolism.* 2010;298:E304-E319.

Rodriguez Annabelle, Muller Denis C., Metter Jeffrey E., et al. Aging, Androgens, and the Metabolic Syndrome in a Longitudinal Study of Aging *J Clin Endocrinol Metab.* 2007;92:3568-3572.

Rogers Nicole H., Witczak Carol A., Hirshman Michael F., Goodyear Laurie J., Greenberg Andrew S.. Estradiol stimulates Akt, AMP-activated protein kinase (AMPK) and TBC1D1/4, but not glucose uptake in rat soleus. *Biochemical and biophysical research communications.* 2009;382:646-650.

Roncari D. A., Van R. L.. Promotion of human adipocyte precursor replication by 17beta-estradiol in culture. *J Clin Invest.* 1978;62:503-508.

Rosner William, Hryb Daniel J., Kahn Scott M., Nakhla Atif M., Romas Nicholas A.. Interactions of sex hormone-binding globulin with target cells. *Molecular and cellular endocrinology.* 2010;316:79-85.

Rubin Gary L., Zhao Ying, Kalus Allan M., Simpson Evan R.. Peroxisome Proliferator-activated Receptor gamma Ligands Inhibit Estrogen Biosynthesis in Human Breast Adipose Tissue: Possible Implications for Breast Cancer Therapy *Cancer Res.* 2000;60:1604-1608.

Ryan A. S., Nicklas B. J., Berman D. M.. Hormone replacement therapy, insulin sensitivity, and abdominal obesity in postmenopausal women. *Diabetes Care.* 2002;25:127-133.

Samarajeewa Nirukshi U., Ham Seungmin, Yang Fangyuan, Simpson Evan R., Brown Kristy A.. Promoter-specific effects of metformin on aromatase transcript expression. *Steroids.* 2011;76:768-771.

Sandeep Thekkepat C., Andrew Ruth, Homer Natalie Z., Andrews Robert C., Smith Ken, Walker Brian R.. Increased In Vivo Regeneration of Cortisol in Adipose Tissue in Human Obesity and Effects of the 11beta-Hydroxysteroid Dehydrogenase Type 1 Inhibitor Carbenoxolone *Diabetes.* 2005;54:872-879.

Santen R. J., Demers L., Ohorodnik S., et al. Superiority of gas chromatography/tandem mass spectrometry assay (GC/MS/MS) for estradiol for monitoring of aromatase inhibitor therapy. *Steroids.* 2007;72:666-671.

Sato Takashi, Matsumoto Takahiro, Yamada Takashi, Watanabe Tomoyuki, Kawano Hirotaka, Kato Shigeaki. Late onset of obesity in male androgen receptor-deficient (AR KO) mice. *Biochemical and biophysical research communications.* 2003;300:167-171.

Schroeder E. T., Zheng L., Ong M. D., et al. Effects of androgen therapy on adipose tissue and metabolism in older men. *J Clin Endocrinol Metab.* 2004;89:4863-4872.

Schroeder E. Todd, Zheng Ling, Yarasheski Kevin E., et al. Treatment with oxandrolone and the durability of effects in older men *J Appl Physiol.* 2004;96:1055-1062.

Scottish Diabetes Survey, Monitoring Group, Scottish Diabetes Study 2008, 2009.

Seidell J. C., Björntorp P., Sjöström L., Kvist H., Sannerstedt R.. Visceral fat accumulation in men is positively associated with insulin, glucose, and C-peptide levels, but negatively with testosterone levels. *Metabolism.* 1990;39:897-901.

Selvin Elizabeth, Feinleib Manning, Zhang Lei, et al. Androgens and Diabetes in Men: Results from the Third National Health and Nutrition Examination Survey (NHANES III) *Diabetes Care.* 2007;30:234-238.

Shimodaira Masanori, Nakayama Tomohiro, Sato Naoyuki, et al. Association study of aromatase gene (CYP19A1) in essential hypertension. *International journal of medical sciences.* 2008;5:29-35.

Shin J. H., Hur J. Y., Seo H. S., et al. The ratio of estrogen receptor alpha to estrogen receptor beta in adipose tissue is associated with leptin production and obesity. *Steroids.* 2007;72:592-599.

Shinozaki S., Chiba T., Kokame K., et al. Site-specific effect of estradiol on gene expression in the adipose tissue of ob/ob mice. *Horm Metab Res.* 2007;39:192-196.

Simpson E. R., Ackerman G. E., Smith M. E., Mendelson C. R.. Estrogen formation in stromal cells of adipose tissue of women: induction by glucocorticosteroids. *Proc Natl Acad Sci U S A.* 1981;78:5690-5694.

Simpson Evan R., Clyne Colin, Rubin Gary, et al. AROMATASE—A BRIEF OVERVIEW *Annual Review of Physiology.* 2002;64:93-127.

Simpson E. R., Misso M., Hewitt K. N., et al. Estrogen-the Good, the Bad, and the Unexpected *Endocr Rev.* 2005;26:322-330.

Singh R., Artaza J. N., Taylor W. E., Gonzalez-Cadavid N. F., Bhasin S.. Androgens stimulate myogenic differentiation and inhibit adipogenesis in C3H 10T1/2 pluripotent cells through an androgen receptor-mediated pathway. *Endocrinology.* 2003;144:5081-5088.

Singh Rajan, Artaza Jorge N., Taylor Wayne E., et al. 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.* 2006;147:141-154.

Smith Matthew R.. Changes in fat and lean body mass during androgen-deprivation therapy for prostate cancer *Urology.* 2004;63:742-745.

Sørensen M. B., Rosenfalck A. M., Højgaard L., Ottesen B.. Obesity and sarcopenia after menopause are reversed by sex hormone replacement therapy. *Obesity research.* 2001;9:622-626.

Spratt Daniel I., Morton Jeremy R., Kramer Robert S., Mayo Sara W., Longcope Christopher, Vary Calvin P. H.. Increases in serum estrogen levels during major illness are caused by increased peripheral aromatization *American Journal of Physiology - Endocrinology and Metabolism.* 2006;291:E631-E638.

Stellato R. K., Feldman H. A., Hamdy O., Horton E. S., Mckinlay J. B.. Testosterone, sex hormone-binding globulin, and the development of type 2 diabetes in middle-aged men: prospective results from the Massachusetts male aging study *Diabetes Care.* 2000;23:490-494.

Stubbins Renee E., Holcomb Valerie B., Hong Jina, Núñez Nomelí P.. Estrogen modulates abdominal adiposity and protects female mice from obesity and impaired glucose tolerance. *European journal of nutrition.* 2012;51:861-870.

Sui Xuemei, Church Timothy S., Meriwether Rebecca A., Lobelo Felipe, Blair Steven N.. Uric acid and the development of metabolic syndrome in women and men *Metabolism.* 2008;57:845-852.

Svartberg Johan, Mühlen Denise, Sundsfjord Johan, Jorde Rolf. Waist circumference and testosterone levels in community dwelling men. The Tromsø study. *European journal of epidemiology.* 2004;19:657-663.

Taieb Joëlle, Mathian Bruno, Millot Françoise, et al. Testosterone Measured by 10 Immunoassays and by Isotope-Dilution Gas Chromatography–Mass Spectrometry in Sera from 116 Men, Women, and Children *Clinical Chemistry.* 2003;49:1381-1395.

Takeda K., Toda K., Saibara T., et al. Progressive development of insulin resistance phenotype in male mice with complete aromatase (CYP19) deficiency. *The Journal of endocrinology.* 2003;176:237-246.

Taponen Saara, Martikainen Hannu, Jarvelin Marjo-Riitta, et al. Hormonal Profile of Women with Self-Reported Symptoms of Oligomenorrhea and/or Hirsutism: Northern Finland Birth Cohort 1966 Study *J Clin Endocrinol Metab.* 2003;88:141-147.

Tchernof A., Després J. P., Dupont A., et al. Relation of steroid hormones to glucose tolerance and plasma insulin levels in men. Importance of visceral adipose tissue. *Diabetes Care.* 1995;18:292-299.

Tchernof Andre, Labrie Fernand, Belanger Alain, et al. Androstane-3alpha,17beta-Diol Glucuronide as a Steroid Correlate of Visceral Obesity in Men *J Clin Endocrinol Metab.* 1997;82:1528-1534.

Tchernof Andre, Desmeules Annie, Richard Christian, et al. Ovarian Hormone Status and Abdominal Visceral Adipose Tissue Metabolism *J Clin Endocrinol Metab.* 2004;89:3425-3430.

Thörne A., Lönnqvist F., Apelman J., Hellers G., Arner P.. A pilot study of long-term effects of a novel obesity treatment: omentectomy in connection with adjustable gastric banding. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity.* 2002;26:193-199.

Tiano Joseph P., Delghingaro-Augusto Viviane, Le May Cedric, et al. Estrogen receptor activation reduces lipid synthesis in pancreatic islets and prevents β cell failure in rodent models of type 2 diabetes. *The Journal of clinical investigation.* 2011;121:3331-3342.

Tran Thien T., Yamamoto Yuji, Gesta Stephane, Kahn C. Ronald. Beneficial effects of subcutaneous fat transplantation on metabolism. *Cell metabolism.* 2008;7:410-420.

Tsai Elaine C., Matsumoto Alvin M., Fujimoto Wilfred Y., Boyko Edward J.. Association of Bioavailable, Free, and Total Testosterone With Insulin Resistance: Influence of sex hormone-binding globulin and body fat *Diabetes Care.* 2004;27:861-868.

T'Sjoen Guy G., Giagulli Vito A., Delva Hans, Crabbe Patricia, De Bacquer Dirk, Kaufman Jean-Marc. Comparative Assessment in Young and Elderly Men of the Gonadotropin Response to Aromatase Inhibition *The Journal of Clinical Endocrinology & Metabolism.* 2005;90:5717-5722.

Turgeon Judith L., Carr Molly C., Maki Pauline M., Mendelsohn Michael E., Wise Phyllis M.. Complex Actions of Sex Steroids in Adipose Tissue, the Cardiovascular System, and Brain: Insights from Basic Science and Clinical Studies *Endocr Rev.* 2006;27:575-605.

Vanbillemont Griet, Lapauw Bruno, Bogaert Veerle, et al. Sex hormone-binding globulin as an independent determinant of cortical bone status in men at the age of peak bone mass. *The Journal of clinical endocrinology and metabolism.* 2010;95:1579-1586.

Van Gaal Luc F., Mertens Ilse L., De Block Christophe E.. Mechanisms linking obesity with cardiovascular disease *Nature.* 2006;444:875-880.

Van Harmelen V., Reynisdottir S., Eriksson P., et al. Leptin secretion from subcutaneous and visceral adipose tissue in women. *Diabetes.* 1998;47:913-917.

Van Londen G. J., Perera S., Vujevich K., et al. The impact of an aromatase inhibitor on body composition and gonadal hormone levels in women with breast cancer. *Breast cancer research and treatment.* 2011;125:441-446.

Van Pelt R. E., Jankowski C. M., Gozansky W. S., Schwartz R. S., Kohrt W. M.. Lower-Body Adiposity and Metabolic Protection in Postmenopausal Women *J Clin Endocrinol Metab.* 2005;90:4573-4578.

Van Pelt R. E., Gozansky W. S., Hickner R. C., Schwartz R. S., Kohrt W. M.. Acute modulation of adipose tissue lipolysis by intravenous estrogens. *Obesity (Silver Spring).* 2006;14:2163-2172.

Van Poznak Catherine, Makris Andreas, Clack Glen, Barlow DavidH, Eastell Richard. Lipid profiles within the SABRE trial of anastrozole with and without risedronate *Breast Cancer Research and Treatment.* 2012;134:1141-1147.

Vermeulen Alex, Verdonck Lieve, Kaufman Jean M.. A Critical Evaluation of Simple Methods for the Estimation of Free Testosterone in Serum *J Clin Endocrinol Metab.* 1999;84:3666-3672.

Wajchenberg B. L.. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocrine reviews.* 2000;21:697-738.

Wake Deborah J., Stimson Roland H., Tan Garry D., et al. Effects of peroxisome proliferator-activated receptor-alpha and -gamma agonists on 11beta-hydroxysteroid dehydrogenase type 1 in subcutaneous adipose tissue in men. *The Journal of clinical endocrinology and metabolism.* 2007;92:1848-1856.

Wake, Deborah J., Strand, et al. Intra-adipose sex steroid metabolism and body fat distribution in idiopathic human obesity *Clinical Endocrinology.* 2007;66:440-446.

Wallace Ian R., McKinley Michelle C., Bell Patrick M., Hunter Steven J.. Sex hormone binding globulin and insulin resistance *Clin Endocrinol.* 2013;78:321-329.

Weisberg Stuart P., McCann Daniel, Desai Manisha, Rosenbaum Michael, Leibel Rudolph L., Ferrante Anthony W.. Obesity is associated with macrophage accumulation in adipose tissue. *The Journal of clinical investigation.* 2003;112:1796-1808.

Woodhouse Linda J., Gupta Nidhi, Bhasin Meenakshi, et al. Dose-Dependent Effects of Testosterone on Regional Adipose Tissue Distribution in Healthy Young Men *J Clin Endocrinol Metab.* 2004;89:718-726.

Wu-Peng S., Rosenbaum M., Nicolson M., Chua S. C., Leibel R. L.. Effects of exogenous gonadal steroids on leptin homeostasis in rats. *Obesity research.* 1999;7:586-592.

Yahyaoui R., Esteva I., Haro-Mora J. J., et al. Effect of long-term administration of cross-sex hormone therapy on serum and urinary uric acid in transsexual persons. *The Journal of clinical endocrinology and metabolism.* 2008;93:2230-2233.

Yanes Licy L., Reckelhoff Jane F.. Postmenopausal Hypertension *American Journal of Hypertension.* 2011;24:740-749.

Yang K., Khalil M. W., Strutt B. J., Killinger D. W.. 11[beta]-Hydroxysteroid dehydrogenase 1 activity and gene expression in human adipose stromal cells: Effect on aromatase activity *The Journal of Steroid Biochemistry and Molecular Biology.* 1997;60:247-253.

Yialamas Maria A., Dwyer Andrew A., Hanley Erin, Lee Hang, Pitteloud Nelly, Hayes Frances J.. Acute Sex Steroid Withdrawal Reduces Insulin Sensitivity in Healthy Men with Idiopathic Hypogonadotropic Hypogonadism *J Clin Endocrinol Metab.* 2007;92:4254-4259.

Yusuf Salim, Hawken Steven, Ôunpuu Stephanie, et al. Obesity and the risk of myocardial infarction in 27 000 participants from 52 countries: a case-control study *The Lancet.* 2005;366:1640-1649.

Zang H., Carlström K., Arner P., Hirschberg A. L.. Effects of treatment with testosterone alone or in combination with estrogen on insulin sensitivity in postmenopausal women. *Fertil Steril.* 2006;86:136-144.

Zang H., Rydén M., Wåhlen K., Dahlman-Wright K., Arner P., Lindén Hirschberg A.. Effects of testosterone and estrogen treatment on lipolysis signaling pathways in subcutaneous adipose tissue of postmenopausal women. *Fertil Steril.* 2007;88:100-106.

Zhao Y., Mendelson C. R., Simpson E. R.. Characterization of the sequences of the human CYP19 (aromatase) gene that mediate regulation by glucocorticoids in adipose stromal cells and fetal hepatocytes. *Mol Endocrinol.* 1995;9:340-349.

Zhao Y., Agarwal V. R., Mendelson C. R., Simpson E. R.. Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene. *Endocrinology.* 1996;137:5739-5742.

Zmuda J. M., Fahrenbach M. C., Younkin B. T., et al. The effect of testosterone aromatization on high-density lipoprotein cholesterol level and postheparin lipolytic activity. *Metabolism.* 1993;42:446-450.

Appendix

Supplemental information (Chapter 5)

	Baseline	Placebo	p
Total cholesterol (mmol/L)	4.5	4.1	0.04
Triglyceride (mmol/L)	1.7	1.1	0.003
HDL cholesterol (mmol/L)	1.21	1.08	0.007
LDL cholesterol (mmol/L)	2.5	2.5	0.6

Although baseline (screening) cholesterol profiles were measured, these were obtained at varying times and in a non-fasted state. Consequently, a comparison between baseline and the anastrozole treatment phase was not regarded as valid. This is confirmed by comparison of baseline samples and placebo phase samples. The significant differences in total cholesterol, triglyceride and HDL cholesterol (table) are highly likely to be accounted for by differences in sampling conditions.