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Metabolic effects of 5α-reductase inhibition in humans

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Doctor of Philosophy

The University of Edinburgh

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Declaration

I declare that this thesis is my own work; I wrote it myself, and the work presented is my own, with the following exceptions:

1. Dr Katherine A Hughes (Endocrinology Unit, University of Edinburgh) gained initial approvals (including ethics and R&D) for the clinical study presented in this thesis.
2. Recruitment of the first 4 volunteers was with assistance from Ms Claire Anderson (Endocrinology Unit, University of Edinburgh) and Mr Norman Todd (Urology, Western General Hospital, NHS Lothian). Dr Rebecca M Reynolds (Endocrinology Unit, University of Edinburgh) was the independent contact for volunteers.
3. Informal advice regarding interim analysis was received from Professor Jane E Norman (Centre for Reproductive Health, University of Edinburgh). Informal advice regarding statistical analysis was received from Professor Gordon D Murray (Centre for Population Health Sciences, University of Edinburgh).
4. Primary care recruitment was conducted with the assistance of the Scottish Primary Care Research Network.

5. Processing and quantitation of MRI spectroscopy data was performed by Dr Calum D Gray (Clinical Research Imaging Centre, University of Edinburgh) and Professor Ian Marshall (Centre for Clinical Brain Sciences, University of Edinburgh).
6. Clinical reporting of MRI scans was performed by Dr Fiona C Minns and colleagues (Radiology, Western General Hospital, NHS Lothian)
7. Extraction of plasma tracer samples, urinary glucocorticoids and plasma dexamethasone samples was performed by Mr Sanjay Kothiya (Endocrinology Unit, University of Edinburgh).
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9. d9-Finasteride was synthesised by Dr Gregorio Naredo (Mass Spectrometry Core, Wellcome Trust Clinical Research Facility, University of Edinburgh). FTICRMS was performed by Mr Diego Cobice (Endocrinology Unit, University of Edinburgh).

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

____

Rita Upreti, Edinburgh, February 2013

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Abstract

5α-reductases (5αRs) catalyse reduction of 4-pregnene steroids, most notably the androgen testosterone to its more potent metabolite dihydrotestosterone (DHT). Well-characterised isozymes of 5αR are designated 5αR1 and 5αR2. Inhibitors of 5αR, finasteride (a 5αR2 inhibitor) and dutasteride (a dual 5αR1 and 5αR2 inhibitor), are utilised in conditions where a reduction in androgen action is desired, including benign prostatic hyperplasia. Although 5αR2 is predominantly expressed in reproductive tissues, both isozymes, but particularly 5αR1, are expressed in metabolic tissues including liver and adipose and both metabolise glucocorticoids as well as androgens; therefore inhibition of 5αR may have consequences for metabolic health. This thesis addresses the hypotheses that 5αR1 inhibition with dutasteride decreases insulin sensitivity and causes dysregulation of the HPA axis in humans.

Metabolism and the HPA axis were studied in men prior to and following 3 months of dutasteride (0.5 mg daily; *n=16*), finasteride (5 mg daily; *n=16*) or control (tamsulosin MR; 0.4 mg daily; *n=14*). Glucose disposal during hyperinsulinaemia was the primary endpoint, measured during a hyperinsulinaemic euglycaemic clamp, with d2-glucose and d5-glycerol tracers. Peripheral insulin sensitivity for both glucose uptake and NEFA suppression

decreased with dutasteride versus both finasteride and control, while hepatic insulin sensitivity was preserved. Body fat increased with dutasteride, though was not accompanied by changes in metabolic or inflammatory gene transcript abundance in subcutaneous adipose biopsies, nor any differences in abdominal adipose depots on post-treatment MRI. Subtle dysregulation of the HPA axis was evident with both 5αR inhibitors, though to a greater degree with dutasteride and changes were largely compensated for.

In support of this study, this thesis also describes the development, validation and application of two novel liquid chromatography tandem mass spectrometry assays; establishing compliance by measuring serum drug levels, and demonstrating effects of 5αR inhibitors on androgen metabolism and adrenal steroidogenesis by measurement of testosterone, DHT and androstenedione.

In conclusion, 5αR1 inhibition with dutasteride, but not finasteride, induces peripheral insulin resistance and increases body fat. Findings presented may have important implications for patients prescribed dutasteride for benign prostatic hyperplasia.

Publications from this thesis

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- Inhibition of 5α-reductase type 1 with dutasteride impairs insulin sensitivity. British Endocrine Societies, Harrogate, 15-18 March 2013
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    - *Awarded Sir James Black prize for best oral presentation*

Poster presentations

- British Endocrine Societies, Harrogate, 19-21 March 2012
- British Mass Spectrometry Society, Cardiff, 11-14 September 2011

Abbreviations

A	Deoxycorticosterone
ACTH	Adrenocorticotrophic hormone
AKR	Aldo-keto reductase
amu	Atomic mass unit
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AP-1	Activating protein 1
APCI	Atmospheric pressure chemical ionisation
AR	Androgen receptor
B	Corticosterone
BA	Butyl acetate
BAT	Brown adipose tissue
BEH	Ethylene bridged hybrid
BMI	Body mass index
BP	Blood pressure
BPH	Benign prostatic hyperplasia
BSA	Body surface area
C18	Carbon 18
CBG	Corticosteroid binding globulin
cDNA	Complementary DNA

CE	Collision energy
CI	Confidence interval
CNS	Central nervous system
Cp	Crossing point
CRH	Corticotrophin releasing hormone
CRP	C-Reactive protein
CRPC	Castration resistant prostate cancer
CSO	Chief Scientist's Office
CT	Computerised tomography
CTIMP	Clinical trial of investigational medicinal product
CV	Coefficient of variation
CXP	Cell exit potential
CYP	Cytochrome P450 enzyme
d	Deuterium
DCM	Dichloromethane
DEE	Diethyl ether
DEPC	Diethylpyrocarbonate
DEXA	Dual-energy x-ray absorptiometry
DH	Dihydro-
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DHT	Dihydrotestosterone

DMC	Data monitoring committee
DNA	Deoxyribonucleic acid
DP	Declustering potential
E	Cortisone
E2	Oestradiol
EA	Ethyl acetate
EC_{50}	Half maximal effective concentration (dose)
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Oestrogen receptor
ESI	Electrospray ionisation
F	Cortisol
FA	Formic acid
GC-MS	Gas chromatography mass spectrometry
GC-MS/MS	Gas chromatography tandem mass spectrometry
GLUT4	Glucose transporter type 4
GnRH	Gonadotrophin releasing hormone
GP	General practitioner
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GTT	Glucose tolerance test
HbA1c	Haemoglobin A1c (glycosylated haemoglobin)

HDL	High-density lipoprotein
hGR	Human glucocorticoid receptor
HLB	Hydrophilic lipophilic balance
HOMA-IR	Homeostatic model assessment of insulin resistance
HPA	Hypothalamic pituitary adrenal
HPLC	High performance liquid chromatography
hs-CRP	High sensitivity CRP
HSD	Hydroxysteroid dehydrogenase
HMW	High molecular weight
IC_{50}	Half maximal inhibitory concentration
IDEAL	Iterative decomposition of water and fat with echo asymmetry and least-squares estimation
IL	Interleukin
IM	Intra-muscular
IR_{AR}	Insulin resistance adiponectin resistin index
IS	Internal standard
IV	Intravenous
k	kilo (x 10^3)
kBq	kilobecquerel
kDa	kilo Daltons
Kg	Kilogram
K_m	Michaelis constant

L	Litres
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDL	Low-density lipoproteins
LLE	Liquid liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
LUTS	Lower urinary tract symptoms
m	milli (x 10^{-3})
M	mol/L
M value	Mean glucose infusion rate at steady state
m/z	Mass to charge ratio
MCP-1	Monocyte chemoattractant protein 1
MHRA	Medicine and Healthcare products Regulatory Agency
MO-TMS	Methoxime-trimethylsilyl
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MRS	Magnetic resonance spectroscopy
MS	Mass spectrometry
MTBE	Methyl *tert*-butyl ether
MW	Molecular weight

n	nano (x 10^{-6})
N	Number
N/A	Not applicable
NADH	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor kappa B
NHS	National health service
NR	Not reported
OFN	Oxygen-free nitrogen
OH	Hydroxy
OHase	Hydroxylase
p	pico (x 10^{-9})
PAR	Pregnane activated receptor
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PFP	Pentafluorophenyl
pKa	Negative log of acid dissociation constant
pH	Negative log of hydrogen ion concentration
POMC	Pro-opiomelanocortin
PRESS	Point-resolved spectroscopy sequence

prn	Pro re nata (as needed)
psi	Pounds per square inch
PSA	Prostate specific antigen
PXR	Pregnane X receptor
QUICKI	Quantitative insulin sensitivity check index
R	Reductase
R&D	Research & Development
Ra	Rate of appearance
RCT	Randomised controlled trial
Rd	Rate of disappearance
RIA	Radioimmunoassay
RIE	Royal Infirmary of Edinburgh
RNA	Ribonucleic acid
RSD	Relative standard deviation
R_t	Retention time
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
SAME	Syndrome of apparent mineralocorticoid excess
SARM	Selective androgen receptor modulator
SD	Standard deviation
SEM	Standard error of the mean
SHBG	Sex hormone binding globulin

SIGN	Scottish Intercollegiate Guidelines Network
SLE	Supported liquid extraction
SNP	Single nucleotide polymorphism
SPCRN	Scottish Primary Care Research Network
SPE	Solid phase extraction
SST	Short Synacthen test
SXR	Steroid and xenobiotic receptor
$T_{1/2}$	Half life
Tamsulosin MR	Modified release tamsulosin
TAT	Tyrosine aminotransferase
TBE	Tris base, boric acid, EDTA
TEA	Triethanolamine
TF	Transcription factor
TG	Triglyceride
TH	Tetrahydro-
TMB	Tetramethylbenzidine
TMSI	Trimethylsilylimidazole
TTR	Tracer tracee ratio
UDP-GT	Uridine diphosphate glucuronyl transferase
UPL	Universal probe library
UPLC	Ultra performance liquid chromatography
USFDA	United States Food and Drug Administration

V	Volts
V/V	Volume for volume
W/V	Weight for volume
WGH	Western General Hospital
WHR	Waist to hip ratio
WTCRF	Wellcome Trust Clinical Research Facility

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Chapter 1: Introduction

1.1 5α-Reductases

5α-Reductases (3-oxo-5α-steroid 4 dehydrogenase, E.C. 1.3.99.5) are enzymes catalysing one of the principal routes of metabolism for steroids with 3-keto-enol functional groups in the A ring (Figure 1.1). Most notably, 5α-reductases (5αRs) catalyse the conversion of the androgen testosterone to its more potent metabolite dihydrotestosterone. Of particular relevance to this thesis, in addition to testosterone, 5αRs also catalyse the irreversible and rate-limiting step of glucocorticoid metabolism (in humans, the metabolism of cortisol to 5α-dihydrocortisol), thus potentially playing a key role in regulating tissue exposure to glucocorticoids.

Three isozymes of 5α-reductase (5αR) have been identified to date, with types 1 and 2 the best characterised at present. 5αRs are widely expressed; 5αR2 is predominantly expressed in the reproductive tract, while both 5αR1 and 5αR2 are expressed in non-reproductive sites, including metabolic tissues such as the liver. 5αRs are the target of pharmaceutical inhibitors used in the treatment of androgen-dependent disorders including benign prostatic hyperplasia, prostate cancer and polycystic ovarian syndrome; 5αR2 is inhibited by finasteride and dual 5αR1 and 5αR2 isozyme inhibition is achieved with dutasteride. This thesis will focus on the effects of 5αR inhibition on glucocorticoid metabolism and consequences for fuel homeostasis and the stress response.

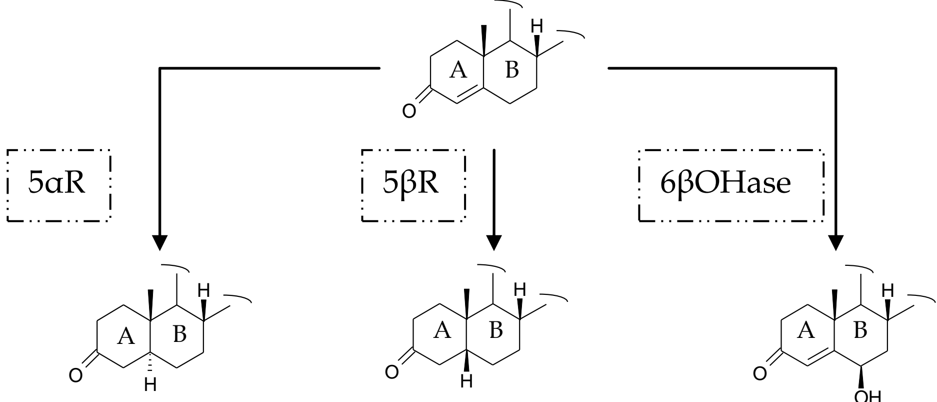

Figure 1.1 Generic schematic of principal metabolic pathways for steroids with 3-keto-enol functional groups in the steroid A ring. R, Reductase; OHase, hydroxylase.

1.1.1 Overview

5αR enzymes were initially described and characterised in the 1950s and 1960s. Early rodent work revealed the presence of an enzyme, most abundantly present in liver, which catalysed the 5α-reduction of steroids (Forchielli et al., 1958, Schneider, 1952, Schneider and Horstmann, 1951). The 1935 isolation of the 'male' steroid hormone, testosterone (Butenandt and Hanisch, 1935, Ruzicka et al., 1935) sparked a flurry of research into androgen synthesis, action, metabolism, and related therapeutic applications (Freeman et al., 2001) – this interest continues unabated today. Initially felt to be an inactive metabolite, 5α-dihydrotestosterone (referred to as DHT throughout this thesis), the 5α-reduced metabolite of testosterone, was later shown to be a more potent androgen. The significance of DHT (and thus of

the enzyme catalysing its formation) was illustrated via two main investigative pathways (Russell and Wilson, 1994). Firstly, the demonstration that DHT was formed in androgen target tissues such as the prostate (Bruchovsky and Wilson, 1968a). Secondly, showing that DHT is crucial to normal virilisation in the developing embryo. Lack of 5αR activity causing a disorder of sexual development was described in the 1970s (Fisher et al., 1978, Imperato-McGinley et al., 1974, Walsh et al., 1974). This condition, now termed 5αR deficiency, occurs in chromosomally 46 XY individuals and is characterised by lack of, or reduction in virilisation at birth, followed by development of a male phenotype at puberty, though without associated prostatic growth.

Russell and colleagues have led much of the research towards characterising the 5αRs. The first cDNA clone of 5αR (Andersson and Russell, 1990) was followed by the finding that, in fact, individuals with 5αR deficiency had normal levels of the only gene known at the time encoding for 5αR (Jenkins et al., 1992). This isozyme was designated to be 5α-reductase type 1 (5αR1), and provided the impetus to pursue the identity of what must be another isozyme influencing androgen action in man. Further work (Andersson et al., 1991) identified the 5αR isozyme most abundant and physiologically significant in the male genital tract, now known as 5α-reductase type 2 (5αR2). Those deficient in this isozyme have a phenotype of

pseudohermaphroditism (Andersson et al., 1991) and while a rat model with inhibition of 5αR2 has been shown to be a phenocopy (George and Peterson, 1988), a transgenic model in mice lacking 5αR2 had only smaller prostates and seminal vesicles, but otherwise normal external genitalia and fertility (Mahendroo et al., 2001).

The discovery of at least two isozymes of 5αR has had significant implications for understanding of steroid physiology and metabolism, and has been key in the therapeutic approach to many androgen target tissue disorders, particularly benign prostatic hypertrophy and androgenic alopecia. The identification of a third 5αR isozyme has led to further diversification of 5αR research. While expression (Godoy et al., 2011, Uemura et al., 2008) and pharmacological inhibition (Kazutoshi et al., 2008, Mohler et al., 2011) of 5αR3 have been described, at present there is very limited understanding of this isozyme, its substrates and its products. This thesis will focus on the roles of 5αR1 and 5αR2, and implications of inhibiting these isozymes in humans.

1.1.2 Biochemistry

5αRs are hydrophobic, membrane-bound enzymes with predicted molecular weights of approximately 28-29 kDa (Russell and Wilson, 1994). Human 5αR1 is a 259 amino acid protein, while human 5αR2 is a 254 amino acid

protein. In human and rat tissues the pH optima allows biochemical differentiation between these two isozymes, with 5αR1 having a pH optima spanning pH 6 - 8.5 and 5αR2 having a pH optima around 5 (Russell and Wilson, 1994). However, the biochemical properties of the type 2 isozyme are not fully defined for all conditions and all species, and it may well function at a neutral pH within the cell (Russell and Wilson, 1994) as this is what is suggested in cell lysate experiments. The location of both 5αR1 and 5αR2 varies by species and tissue, with rat prostate (type 1) and human prostate (type 2) in a perinuclear location, and rat liver (type 1) and human liver (type 2) located in endoplasmic reticulum (Russell and Wilson, 1994). Half-lives of both enzymes are in the order of 20-30 hours (Russell and Wilson, 1994).

1.1.3 Genetics

In humans, 5αR1 is encoded for on chromosome 5 (*SRD5A1*) (Jenkins et al., 1991) and 5αR2 on chromosome 2 (*SRD5A2*) (Labrie et al., 1992). Both isozymes have five exons and four introns, and retain a high degree of homology between isozymes and species, suggestive of a common origin (Russell and Wilson, 1994). There is also an apparently non-functioning pseudogene mapped to the X chromosome (Jenkins et al., 1991). Genetic abnormalities in 5αR have been the initial driving focus of much of the research around 5αRs. Details of genetic mutations causing pseudohermaphroditism have been reviewed (Russell and Wilson, 1994).

Additional genetic variants have also been described in diseases with metabolic and hormonal dyshomeostasis. In polycystic ovarian syndrome (PCOS), SNPs in both 5αR genes are associated; a variant in *SRD5A2* is protective against PCOS, while several different variants in *SRD5A1* are associated with presence, and increased severity, of hirsutism (Goodarzi et al., 2006). In benign prostatic hyperplasia, single nucleotide polymorphisms (SNPs) in *SRD5A1* (though not *SRD5A2*) are associated with disease severity (Klotsman et al., 2004), however implications of this are not well understood at present. An increased risk of developing the metabolic syndrome is associated with a SNP in *SRD5A2* in those previously treated for testicular cancer (Boer et al., 2011). Peripheral arterial disease has been associated with two SNPs in *SRD5A1,* though not with *SRD5A2* (Signorelli et al., 2008); given the previous association of these SNPs with decreased conversion of testosterone to DHT, it was concluded that decreased local metabolism of testosterone associated with the *SRD5A1* SNPs may confer increased risk of peripheral arterial disease. In a study of 57 males with type 2 diabetes, a SNP associated with increased 5αR1 activity (seen as an increase in DHT/testosterone ratio) has been identified, however no genetic polymorphisms in either *SRD5A1* or *SRD5A2* were associated with the diabetic phenotype (Ellis et al., 2005).

While several SNPs in both *SRD5A1* and *SRD5A2* have been identified, and in future may provide further understanding of disease mechanisms and exciting opportunities for targeted therapeutics, these findings have not yet been translated into clinical practice.

1.1.4 Tissue distribution

5αR1 and 5αR2 are widely expressed in many species (summarised for human, mouse and rat in Table 1-1), and an understanding of expression patterns for both isozymes is paramount to understanding enzyme function. Research pertaining to 5αRs has been predominantly conducted in rodent models, however there are important inter-species differences in the tissue distribution of the 5αR isozymes. These differences are relevant to interpretation of which tissues will be affected by genetic manipulation (animal models), genetic defects (humans) and consequences of pharmacological inhibition. In humans, 5αR1 exists primarily in non-genital skin, whereas 5αR2 is found primarily in the reproductive tract including prostate, seminal vesicles, epididymis and genital skin (Russell and Wilson, 1994). Both isozymes are found in liver (Russell and Wilson, 1994) and while hepatic 5αR1 is thought to be the key enzyme in glucocorticoid metabolism, at this stage the differential role played by 5αR1 versus 5αR2 in human liver is not well defined. In mouse prostate 5αR2 predominates, in contrast to rat prostate where both isozymes can be detected (Russell and Wilson, 1994). In

contrast to human, where both isozymes are expressed, hepatic 5αR expression in rats is solely type 1, with female rats expressing 10-20 times that of male rats (Russell and Wilson, 1994). Otherwise, 5αR expression is similar in males and females. As well as the liver and reproductive tract, 5αRs are also widely expressed in the central nervous system in mammalian, aquatic and avian species (Lephart, 1993a, Rego et al., 2009). Both mRNA and protein have been demonstrated for 5αR3 in several tissues in humans including skin, skeletal muscle, liver, kidney, colon and pancreas, as well as in several malignant tissues (Godoy et al., 2011, Uemura et al., 2008). However, the implications of 5αR3 action are not well understood at present.

Table 1-1 (below): Tissue distribution of 5α-reductases types 1 and 2 in human (H), rat (R) and mouse (M). In cases of differing evidence, those demonstrating expression are shown. 5αR1, 5α-reductase type 1; 5αR2, 5α-reductase type 2; ✓, present; ✗, absent; ±, very low levels; IC, either immunocytochemistry or immunohistochemistry; vasc., vasculature; G, gamma aminobutyric acid; g, glutaminergic neurons; PN, peripheral nerves. Activity[a], demonstration of substrate → product in conditions designed for optimal isozyme action. Activity[b], ex vivo or in vivo demonstration of substrate 5α-reduction . *mRNA identified but isozyme not specified. For many organs expression/activity varies considerably depending on component of tissue tested – details are within referenced papers. An earlier version of this table has been published previously (Nixon et al., 2012).

	5αR1			5αR2			Isozyme unconfirmed	References
Method System	mRNA	Protein/ IC	Activity[a]	mRNA	Protein/ IC	Activity[a]	Activity[b]	
METABOLIC/ CARDIORESPIRATORY								
Liver	H✓; R✓; M✓	H✓; R✓	R✓	H✓; R×; M✓	H✓; R×		H✓	(Russell and Wilson, 1994) (Normington and Russell, 1992) (Mahendroo et al., 1997) (Livingstone et al., 2000a) (Seo et al., 2009) (Houston et al., 1987)
Adipose	H✓; R✓; M✓			H✓; M×			H✓; R✓	(Zyirek et al., 1987) (Wake et al., 2007) (MacKenzie et al., 2008) (Livingstone et al., 2009b) (Drake et al., 2005) (Longcope and Fineberg, 1985)
Skeletal Muscle	H✓; R✓	H✓; R✓		H±; R×	H×		H±	(Thigpen et al., 1993) (Longcope and Fineberg, 1985, Aizawa et al., 2010, Pollanen et al., 2011)
Heart/ vasc.	R×			R×			H✓; R✓	(Normington and Russell, 1992) (Lephart, 1993b) (Blom et al., 2001) (Milewich et al., 1987)
Pancreas							H✓	(Iqbal et al., 1983)
Kidney	H×; R✓	H×	H✓	H×; R✓	H×	H✓	M±	(Russell and Wilson, 1994) (Thigpen et al., 1993) (Mowszowi.I and Bardin, 1974)
Adrenal	H×; R✓; M±	H×; R✓	H✓	H×; R✓; M✓	H×	H×	R✓	(Russell and Wilson, 1994) (Thigpen et al., 1993) (Yokoi et al., 1998b) (Luu-The et al., 2005) (Colby and Kitay, 1972)
Lung	H✓; R✓; M✓	H✓	H✓	H✓; R×	H×			(Normington and Russell, 1992) (Provost and Tremblay, 2007) (Provost et al., 2002) (Kimura et al., 2003)
IMMUNE								
Spleen	R✓			R×				(Normington and Russell, 1992)
Monocytes/ Macrophages	H✓; M×			H±; M×			H✓; R✓; M✓	(Milewich et al., 1983) (Hammer et al., 2005) (Lofthus et al., 1984) (Araneo et al., 1991) (Yang et al., 2011)
Lymphocytes	H✓		H✓	H±			M✓	(Zhou et al., 1998) (Samy et al., 2001)
Thymus	R✓*			R✓*				(Borlak et al., 2004)
SKIN & BONE								
Genital Skin	H✓	H×		H✓	H✓		H✓; R✓	(Russell and Wilson, 1994) (Thiele et al., 2005) (Pinsky et al., 1978) (Dube et al., 1975)
Non-Genital Skin	H✓	H✓		H±	H✓		H✓; R✓	(Russell and Wilson, 1994) (Luuthe et al., 1994) (Takayasu et al., 1980) (Eicheler et al., 1995) (Randall et al., 1982) (Dube et al., 1975)
Hair follicle	H✓	H✓		H✓	H✓		H✓	(Eicheler et al., 1998) (Eicheler et al., 1995) (Sawaya and Price, 1997)
Sweat gland	H✓							(Sato et al., 1998)
Cartilage	R✓		R:♂✓,♀×	R×				(Raz et al., 2005)
Bone	H✓		H✓	H✓		H±	R×	(Turner et al., 1990) (Issa et al., 2002)

SENSORY								
Olfactory bulb	R✓; M±(G); M✓(g)	R✓; M±(G); M✓(g)					R✓	(Agis-Balboa et al., 2006) (Kiyokage et al., 2005) (Li et al., 1997)
Eyes	H✓			H✓			H✓	(Weinstein et al., 1991) (Rocha et al., 2000)
NEUROLOGICAL								
Hypothalamus	H✓; R✓; M✓	H✓; R✓	R✓	H×; R×	H×		R✓	(Karolczak et al., 1998) (Russell and Wilson, 1994) (Thigpen et al., 1993) (Pelletier et al., 1994) (Lephart, 1993b) (Li et al., 1997)
Pituitary	H×; R✓	H×; R✓	R✓	H×; R×	H×		R✓	(Russell and Wilson, 1994) (Nowak, 2002) (Lephart, 1993b) (Yokoi et al., 1996)
Thalamus	M✓(G, g)	R✓; M✓(G,g)					R✓	(Agis-Balboa et al., 2006) (Pelletier et al., 1994) (Li et al., 1997)
Hippocampus	H✓; M×(G); M✓(g)	R✓; M×(G); M✓(g)		H×			R✓	(Agis-Balboa et al., 2006) (Pelletier et al., 1994) (Li et al., 1997) (Stoffel-Wagner et al., 2000) (Melcangi et al., 1988)
Cortex	H✓; R✓; M×(G); M✓(g)	H✓; R✓; M×(G); M✓(g)	H✓	H×; R✓	H×		R✓	(Agis-Balboa et al., 2006) (Pelletier et al., 1994) (Torres and Ortega, 2006) (Li et al., 1997) (Stoffel-Wagner et al., 1998) (Steckelbroeck et al., 2001) (Melcangi et al., 1988)
Medulla oblongata	H✓	H✓		H✓	H✓		R✓	(Thigpen et al., 1993) (Li et al., 1997)
Pons	H✓	H×		H×	H×		R✓	(Russell and Wilson, 1994) (Thigpen et al., 1993) (Li et al., 1997) (Melcangi et al., 1988)
Amygdala	M(g)✓	M(g)✓					R✓	(Agis-Balboa et al., 2006) (Li et al., 1997)
Corpus Collosum	M×	M×					R✓	(Agis-Balboa et al., 2006) (Melcangi et al., 1988)
Striatum	M(G)✓	M(G)✓						(Agis-Balboa et al., 2006)
Cerebellum	H✓; M✓(G); M± (g)	H✓; M✓(G); M±(g)		H×	H×		R✓	(Russell and Wilson, 1994) (Thigpen et al., 1993) (Agis-Balboa et al., 2006) (Li et al., 1997) (Melcangi et al., 1988)
Spinal cord	R✓	R✓		R✓	R✓		R✓; M✓	(Pozzi et al., 2003) (Patte-Mensah et al., 2004) (Maclusky et al., 1987) (Hauser et al., 1987)
PN / paraganglia		R✓					R✓	(Yokoi et al., 1998a) (Melcangi et al., 1990)

REPRODUCTIVE								
Prostate	H✓; R✓	H×; R✓	H✓; R✓; M±	H✓; R✓	H✓; R✓	R✓;M✓	H✓	(Shirakawa et al., 2004) (Russell and Wilson, 1994) (Normington and Russell, 1992) (Mahendroo et al., 2001) (Houston et al., 1987)
Testes	H×; R✓	H×	M✓	H✓; R✓	H✓	M×	M✓	(Russell and Wilson, 1994) (Mahendroo et al., 2004) (Sheffield and Oshaughnessy, 1988)
Epididymis	H✓; R✓; M✓	H×; R✓	R✓; M✓	H✓; R✓; M✓	H✓; R✓	R✓; M✓	H✓	(Thigpen et al., 1993) (Mahony et al., 1998) (Delarminat et al., 1980) (Viger and Robaire, 1994) (Normington and Russell, 1992) (Mahendroo et al., 2001)
Vas Deferens	R✓			R✓			H✓; R✓; M✓	(Russell and Wilson, 1994) (Seethalakshmi et al., 1982) (Dupuy et al., 1979) (Jeanfaucher et al., 1986)
Seminal Vesicles	H×; R✓	H×; R✓	M×	H✓; R✓	H✓; R✓	M×		(Pratis et al., 2000) (Russell and Wilson, 1994) (Mahendroo et al., 2001)
Ovary	H✓; R✓; M✓	H×	H✓	H✓; R×; M✓	H×	H✓		(Russell and Wilson, 1994) (Normington and Russell, 1992) (Thigpen et al., 1993) (Jakimiuk et al., 1999) (Backstrom et al., 1986) (Milewich et al., 1995) (Luu-The et al., 2005)
Vagina	H✓; M✓			H✓; M✓			R✓	(Berman et al., 2003) (Blom et al., 2001) (George, 1993) (Luu-The et al., 2005)
Uterus	H✓;M✓	H✓; M✓		H✓; M±	H✓		R✓	(Mahendroo et al., 1997) (Blom et al., 2001) (Luu-The et al., 2005) (Ito et al., 2002)
Placenta	M✓	H✓; M✓	H✓		H✓	H✓	R✓	(Milewich et al., 1979) (Dombroski et al., 1997) (Vu et al., 2009) (Mahendroo et al., 1997) (Chan and Leathem, 1975)
Breast	H✓	H✓		H✓	H✓		H✓; R✓; M✓	(Lloyd, 1979) (Wiebe et al., 2000) (Suzuki et al., 2001) (Mori et al., 1978) (Abulhajj and Kiang, 1982)
GASTRO-INTESTINAL								
Stomach	R✓			R×				(Normington and Russell, 1992)
Intestine	R✓			R✓			H✓; R✓	(Normington and Russell, 1992) (Nienstedt et al., 1980b) (Nienstedt et al., 1980a) (Eiknes et al., 1983)

1.1.5 Substrates and products

Known substrates of 5αR are steroid hormones with a double bond between carbons 4-5 of the A-ring (Figure 1.2).

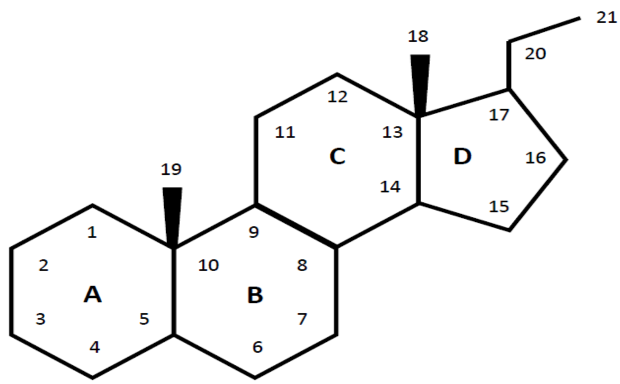

Figure 1.2 Schematic of basic steroidal structure. Steroids have three cyclohexane rings (denoted, by convention, A, B and C) and one cyclopentane ring (denoted D). Individual carbon atoms within the steroid molecule are denoted by numbers. Carbons 4 and 5 of the A ring are the site of 5α-reductase enzyme activity, where a double bond in this position is reduced to form the 5α-dihydrosteroid.

5α-reductases catalyse the irreversible reaction:

3-oxo-δ^4-steroid + 2NADPH → 3-oxo-5α-steroid + $2NADP^+$

The consequence for the substrate is the reduction of the carbon 4-5 double bond, with NADPH acting as cofactor. 5αR has an absolute requirement for NADPH in order to catalyse the A-ring reduction of its steroid substrates, and is unable to utilise NADH as an alternative (Frederiksen and Wilson,

1971). *In vitro* studies suggest it is the denaturing of the 5αR enzyme which leads to the cessation of its reductase actions, rather than paucity of substrate or NADPH (Frederiksen and Wilson, 1971). However, this is in contrast to the findings in other studies, suggesting substrate depletion is responsible for any cessation of 5αR activity (Larsen, 2002).

The metabolic pathway of 3-oxo-δ^4-steroids via 5αR generally follows the pattern:

3-oxo-δ^4-steroid → 5α-dihydrosteroid → 3α (or β), 5α-tetrahydrosteroid

The initial reaction is catalysed by 5αRs and is irreversible, while the latter is catalysed by 3α- or 3β- hydroxysteroid dehydrogenase and is reversible (Figure 1.3). 3αHSDs make up the aldo-keto reductase (AKR) 1C enzyme family, of which there are four known isozymes. AKR1C1, AKR1C2, and AKR1C3 all have a role in the metabolism of androgens and progesterones, with one or more of these detected in liver, prostate, breast, uterus and brain (Penning et al., 2000). AKR1C4 is liver specific and acts together with the 5α and 5β-reductases (Penning et al., 2000). 3αHSD versus 3βHSD metabolite formation varies greatly in an isoform and tissue dependent manner; ratios of 3α:3β HSD metabolites are 1:4 (AKR1C1:3βHSD), 20:1 (AKR1C2:3βHSD), 1.5:1 (AKR1C3:3βHSD), and 3.6:1 (AKR1C4:3βHSD), (Steckelbroeck et al., 2004).

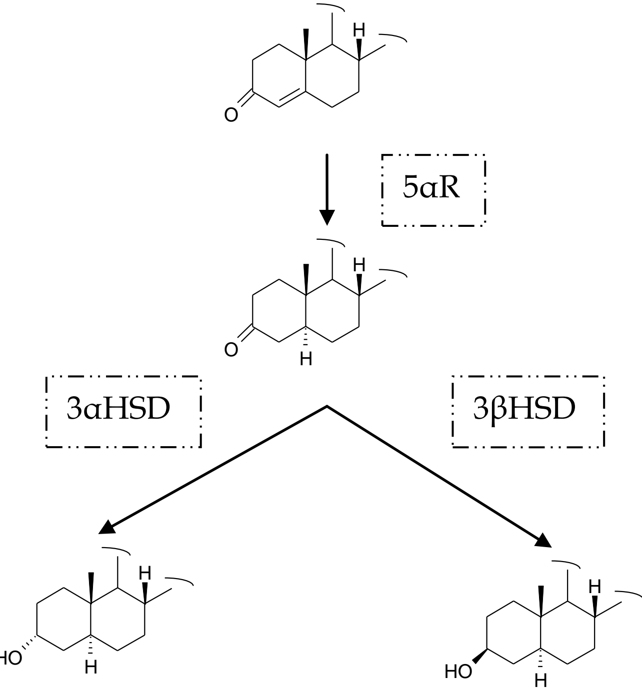

Figure 1.3. Generic metabolic pathways for 3-oxo-δ^4-steroids metabolised by 5α-reductase. Enzymes indicated in interrupted boxes. R, reductase; HSD, hydroxysteroid dehydrogenase.

Many hormonally active steroids, other than oestrogens, have the δ^4-3-oxo group in the A ring, and tetra-hydro reduction of the A ring was traditionally thought to inactivate these steroids prior to excretion (Makin et al., 2010), rendering them more susceptible to conjugation. Prior to the identification of 5αR2, *in vitro* investigations characterised substrate nature and reaction rates for a number of 3-oxo-δ^4-steroids. McGuire and colleagues (McGuire et al., 1960) demonstrated the many substrates of 5αR in rat liver, but also indicated some 3-oxo-δ^4-steroids which were not 5α-reduced. These were either unsaturated at C1-2, or had a methyl substitution at C2 or C6

(McGuire et al., 1960). Another study characterised 5αR substrate specificity of a variety of 3-oxo-δ^4-steroids in rat prostate, and demonstrated the likelihood of a single 5αR isozyme in prostate as compared to liver (Frederiksen and Wilson, 1971).

In humans, the most biologically significant substrates of 5αR are testosterone, progesterone, aldosterone and cortisol (Figure 1.4). Human 5αR (prior to isozyme identification) has been shown to act with differing efficiencies on its key substrates; K_m values for testosterone (3.6 μM), androstenedione (1.7 μM) and progesterone (0.8 μM) demonstrate this (Andersson and Russell, 1990). While catabolic activity with mineralocorticoids and glucocorticoids has been demonstrated (Frederiksen and Wilson, 1971, Andersson and Russell, 1990), their comparative K_m values were unable to be determined. Due to the known biological activity of their metabolites, testosterone and progesterone are certainly the most researched substrates of 5αR, however it is now increasingly evident that the 5α-reduced metabolites of cortisol may also have physiologically relevant effects (McInnes et al., 2004, Yang et al., 2011), and these are discussed further in Section 1.4.1.

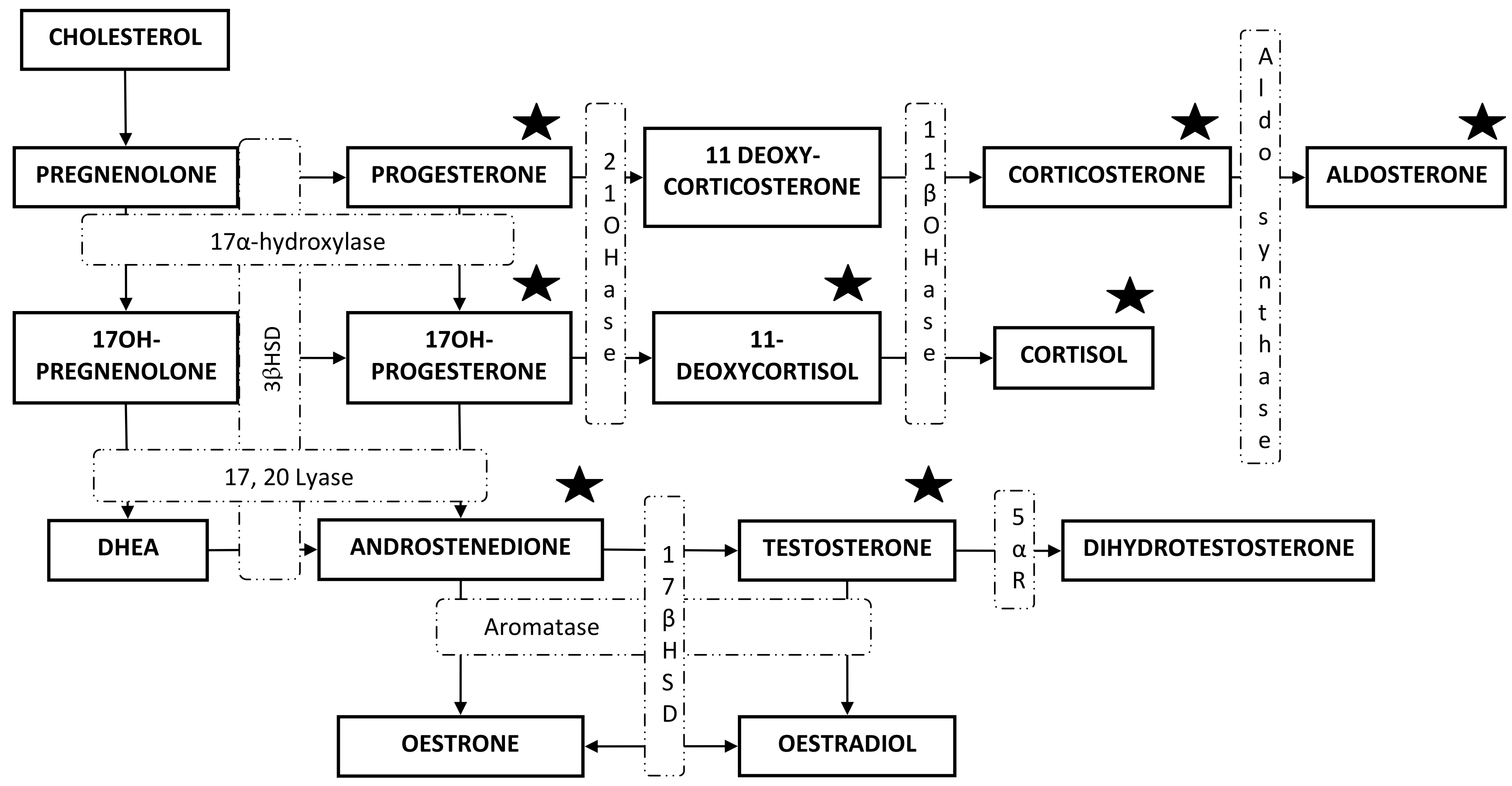

Figure 1.4 Classical pathway of steroid synthesis; substrates of 5α-reductase indicated with stars. All steroid hormones (in solid boxes) are formed from their common precursor cholesterol. Enzymes (in interrupted boxes) catalyse conversion reactions. 3βHSD, 3β-hydroxysteroid dehydrogenase; 21OHase, 21α-hydroxylase; 11βOHase, 11β-hydroxylase; Aldo synthase, aldosterone synthase; 17βHSD, 17β-hydroxysteroid dehydrogenase; 5αR, 5α-reductase.

1.1.5.1 Androgens

Androgens (discussed further in Section 1.21.2) are steroid hormones instrumental in the establishment and maintenance of the male phenotype, and are also key players in many aspects of health (and disease) in both men and women. The conversion of the androgen testosterone to its more potent metabolite 5α-dihydrotestosterone (DHT) is the best characterised, most clinically significant and pharmacologically targeted reaction catalysed by 5αR. 5.6% of plasma testosterone in men, and 3.5% in women is irreversibly converted to DHT (Ito and Horton, 1971). Free DHT levels in men peak during their 20s, with levels slowly declining thereafter (Starka et al., 2009). DHT production patterns in women do vary from men, with the key differences being a reduced amount of testosterone as substrate, and the fact that their DHT is primarily sourced from androstenedione rather than testosterone (Ito and Horton, 1971). The majority of DHT exists as a result of peripheral conversion from testosterone, principally in prostate and skin, rather than direct secretion (Ito and Horton, 1971), however non-classical 'backdoor' pathways of DHT synthesis do exist and are discussed further in Section 1.2.1.

The relative importance of DHT in the developing male is seen both pre- and post-natally. The human placenta is known to express both isozymes of 5αR, and when compared with female fetuses, male fetuses show significantly

greater expression of 5αR1, and a trend towards a greater expression of 5αR2 (Vu et al., 2009). Post-natal significance of DHT is reflected in the peak ratio of DHT to testosterone, which is maximal in infancy and childhood, with rates dropping until approximately age 20 years after which they remain steady into old age (Starka et al., 2009).

Patients with a complete 5αR2 deletion can have normal serum DHT concentrations and present with variable degrees of masculinisation (Imperato-Mcginley et al., 1991, Thigpen et al., 1993), revealing the ability of 5αR1 to deputise for 5αR2. Prior to the identification of the different isozymes, in 5αR mutations leaving very low levels of 5αR activity, administration of exogenous testosterone was shown to increase DHT concentrations from low normal to within the normal range (Price et al., 1984). These observations support the notion of DHT acting as an endocrine hormone, as well as in an autocrine and paracrine manner as was traditionally thought. This also adds weight to the concept of inhibiting both 5αR1 and 5αR2 for disorders such as benign prostatic hypertrophy, as DHT formed in extra-prostatic tissues may be acting in an endocrine manner to drive prostatic growth. Pharmacological 5αR inhibition now forms a key part of the medical armamentarium in treatment of benign prostatic hyperplasia, and is discussed further in Section 1.3.2.

Additional androgen substrates of 5αR are epitestosterone and androstenedione. Epitestosterone, an epimer of testosterone, is a naturally occurring steroid hormone. While presumed to act via both the androgen receptor, as well as non-genomic routes, mechanisms of epitestosterone action are not confirmed (Starka, 2003). *In vitro* radioactive experiments show epitestosterone to be a better 5αR substrate than testosterone, with K_m values approximately 3 times lower (Frederiksen and Wilson, 1971), and it is also able to serve as a competitive inhibitor of testosterone reduction in prostatic nuclei (Frederiksen and Wilson, 1971, Starka, 2003). Less than 5% of epitestosterone is converted to its 5α- (and then 3α-) reduced metabolite, 5α-androstane-3α,17α-diol, prior to urinary excretion (Starka, 2003) and the physiological properties of these metabolites are unknown. Androstenedione can be formed from the peripheral metabolism of DHEA. It is then reduced by either 5αR or 5βR (in equal proportions), followed by 3αHSD reduction to form androsterone and etiocholanolone respectively (Auchus, 2009). In rat prostate, androstenedione was demonstrated to be approximately equal to testosterone in its substrate specificity for 5αR (Frederiksen and Wilson, 1971). The properties of androstenedione's 5α-reduced metabolite are unknown. Androsterone is readily converted into DHT (though is not amenable to aromatisation) (Auchus, 2009), and while briefly of interest in conditions such as hirsutism, it has been shown to be of limited clinical utility at present (Zwicker and Rittmaster, 1993). Both epitestosterone (Piper

et al., 2009) and androsterone (Aguilera et al., 2009) are now most commonly measured as part of doping control efforts in professional sport.

1.1.5.2 Glucocorticoids

Glucocorticoids (discussed further in Section 1.4), steroid hormones synthesised by the adrenal glands, are vital to many aspects of health including glucose regulation and response to stress. The action of 5αR on glucocorticoids is the rate limiting and irreversible step in their degradative pathway (Figure 1.7, Section 1.4.1). As with other 5αR substrates, glucocorticoids are metabolised to their 5α-dihydro and 5α-tetrahydro metabolites. While several glucocorticoids are 5αR substrates, the most significant in humans is cortisol.

Cortisol is reduced to 5α-dihydrocortisol via hepatic 5αR, and is then further reduced prior to urinary excretion, with 5α-tetrahydrocortisol forming between 11-25% urinary cortisol metabolites (Shamim et al., 2000, Andrew et al., 1998, Best and Walker, 1997). While 5αR action on glucocorticoids is predominantly hepatic (Baudrand et al., 2011), it is also thought to metabolise glucocorticoids in adipose (Wake et al., 2007, MacKenzie et al., 2008), possibly the brain (Thigpen et al., 1993), but not in the prostate (Frederiksen and Wilson, 1971). In rodents, corticosterone (B) is the most significant glucocorticoid, owing to a lack of adrenal 17α-hydroxylase, and is

reduced by 5αR to form 5α-dihydrocorticosterone (5αDHB) which is further reduced to 5α-tetrahydrocorticosterone (5αTHB).

Two other glucocorticoids, namely cortisone and 11-deoxycortisol, have been shown to act as 5αR substrates, however have not yet been shown to be of clinical significance. Cortisol can be interconverted with its biologically inactive metabolite cortisone by the actions of the 11βHSD enzymes. While C4-5 reduction of cortisone is predominantly catalysed via 5β-reductase (5βR), it can also be reduced by 5αR to form 5α-dihydrocortisone *in vitro* in microsomal rat liver fractions (McGuire and Tomkins, 1960), though not in rat prostate (Frederiksen and Wilson, 1971). Humans are not known to excrete a urinary 5α-metabolite of cortisone (Bush and Mahesh, 1959). 11-Deoxycortisol is usually converted to cortisol via the actions of the enzyme 11β-hydroxylase. It is known to be approximately as good a substrate as testosterone for 5αR (Frederiksen and Wilson, 1971) and *in vitro* studies in rat liver homogenates (Forchielli et al., 1955) confirm this. There is very little known about the site(s) and nature of in vivo 11-deoxycortisol metabolism in humans. A study with tritiated 11-deoxycortisol in 6 healthy human volunteers (Dehertogh et al., 1964) showed it disappears rapidly from plasma, with only 3% of initial IV dose remaining after 5 minutes, 0.08% remaining after 90 minutes, and 70% excreted in urine within 24 hours, however the relative contribution of 5αR in this metabolism is not defined.

However, the *in vivo* actions of the 5α-reduced metabolites of 11-deoxycortisol remain unknown.

While thought to lack metabolic effects, 5α-reduced glucocorticoids have anti-inflammatory effects and are discussed further in Section 1.4.1.

1.1.5.3 Progesterones

Progesterone is a steroid hormone synthesised by the ovaries and adrenal cortex, classically known for its role in normal and abnormal female reproductive physiology. 40-60% of plasma progesterone is metabolised to 5α-dihydroprogesterone (5αDHP) principally in corpus luteum (Dombroski et al., 1993), placenta (Milewich et al., 1979), liver and brain. Approximately 90% of synthesis and subsequent metabolism of 5αDHP occurs in extra-hepatic sites (Dombroski et al., 1993). Prior to excretion, metabolism preferentially follows a pattern of 3β- (rather than 3α-) reduction, followed by 6α-hydroxylation, to finally be excreted in urine in the form of 3β,6α-dihydroxy-5α-pregnan-20-one (Chantilis et al., 1996).

Recent interest in the 5α-reduction of progesterone has primarily been in the field of psychiatric and neurological research. Progesterone is present at a greater concentrations in the brain (as compared to testosterone), and its kinetic properties potentially make it a more favourable substrate for 5αR

(Lephart, 1993a). 5αR1 is present in substantially larger amounts than 5αR2 in rat, mouse and human brain (Rego et al., 2009), thus is thought to be the most important 5αR isozyme in neurological steroid metabolism.

5αDHP can bind to the progesterone receptor (Dong et al., 2001) and function as a neurosteroid. In male rats, 5αDHP has been shown to be a potent anti-convulsant in a model of complex partial seizures with secondary generalisation (Lonsdale et al., 2006), and shows greater effect than either progesterone or 5αTHP. 5αDHP (along with progesterone and allopregnanolone) can promote Schwann cell proliferation through interaction with other transcription factors, and plays a part in the regulation of myelin formation (Brinton et al., 2008).

There is a substantial body of evidence indicating that the 3αHSD metabolite of 5αDHP, allopregnanolone, is active as a modulator of the GABAergic neurotransmission system, likely via non genomic mechanisms (Purdy et al., 1990). It is the 3α, and not the 3β, metabolites of 5αDHP that have this capability (Purdy et al., 1990). In a murine model, protracted social isolation stress was associated with a substantial decrease in 5αR1 activity, with subsequent reduction in both 5αDHP and allopregnanolone levels (Dong et al., 2001).

While reduced activity of 5αR1 is associated with some negative phenotypes (Dong et al., 2001), the use of 5αR inhibitors in rodent models has demonstrated an ability to reduce potentially distressing phenotypes including compulsive (Bini et al., 2009) and psychotic behaviours (Bortolato et al., 2008). Given the many substrates of 5αR1 and its regional and inter-species variations in brain expression, dissecting the contribution of different 5αR substrates and metabolites is difficult; it is likely that pathways influencing cognition, mood and behaviour are modulated by multiple 5α-reduced neuroactive steroids.

In addition to progesterone, two other progesterones are known to be 5αR substrates, namely 20α-dihydroprogesterone (20αDHP) and 17α-hydroxyprogesterone (17αOHP). 10% of plasma progesterone is converted to 20αDHP (Dombroski et al., 1993), and this can then be converted by 5αR to 20α-hydroxy-5α-pregnan-3-one. In female rats 5αR action on 20αDHP is prominent in the anterior pituitary and medial basal hypothalamus, with metabolite action likely mediated via non-genomic routes due to low affinity for the progesterone receptor (Nowak, 2002). 5α-Reduced metabolites of 20αDHP are hypothesised, though not confirmed, to have a biologically significant role as neurosteroids. 17αOHP is a glucocorticoid precursor, and while *ex vivo* work in human fetal small intestine (Nienstedt et al., 1980b) demonstrated that 17OHP could be 5α-reduced, it was 5β- metabolites which

predominated. While this early work demonstrates intestinal 5α-reducing capacity, these findings have not extended into further published research.

1.1.5.4 Mineralocorticoids

Aldosterone is a substrate for 5αR in the rat kidney, with 5α- and 5β-metabolites formed in approximately equal amounts. Of interest is that the 5α- and 3α,5α- metabolites of aldosterone retain significant mineralocorticoid activity, and though their relative potency is less than aldosterone itself, they may still be biologically relevant (McDermott et al., 1983). These metabolites have been shown to cause hypertension in adrenalectomised spontaneously hypertensive rats, and when compared to 3β, 5α- or 3α,5β- aldosterone, the 5α- or 3α,5α- aldosterone metabolites have a greater propensity to cause hypertension and suppress plasma renin activity (Gorsline et al., 1986).

Another mineralocorticoid, 11-deoxycorticosterone, is a substrate for 5αR (Frederiksen and Wilson, 1971). Both 11-deoxycorticosterone and its 5α-reduced metabolite, 5α-dihydro-11-deoxycorticosterone, show mineralocorticoid activity, though the latter to a lesser degree (Sekihara et al., 1978). 5α-dihydro-11-deoxycorticosterone has also been shown to act as an antagonist to more potent mineralocorticoids (Sekihara and Yazaki, 1993). The physiological relevance of the 5α-reduced mineralocorticoids in humans is unknown.

1.2 Androgens

1.2.1 Significance, Synthesis and Metabolism

Androgens are steroid hormones which play an integral role in development and maintenance of gender phenotypes, and are also key players in other organ systems including bone and metabolism. Androgen production occurs principally in reproductive tissues in males and both the adrenal glands and ovaries in females. The main adrenal androgens are dehydroepiandrosterone (DHEA) and androstenedione, while the main androgens synthesised in extra-adrenal sites are testosterone and dihydrotestosterone (DHT).

The sulfated derivative of dehydroepiandrosterone (DHEAS) is the quantitatively most significant androgen in adult humans (El Kihel, 2012), however while altered concentrations of DHEA and DHEAS are associated with different health conditions, their role and mechanism of action is yet to be well described. Androstenedione is important as a precursor to the extra-adrenal synthesis of testosterone. Synthesis of testosterone and its subsequent metabolism are summarised in Figure 1.5. Testosterone and its more potent metabolite dihydrotestosterone are biologically the most significant androgens and are the focus of androgen discussion in this thesis.

Products of testosterone metabolism include androgens, principally dihydrotestosterone (catalysed by 5αR) and oestrogens (catalysed by aromatase). While ovarian steroid production is the main source of oestrogens in pre-menopausal women, peripheral conversion of androgens to oestrogens is the predominant source in men and post-menopausal women; therefore this pathway provides another important avenue of androgen action, and modulation of any aspects of androgen or oestrogen physiology cannot be viewed in complete isolation. Along with all 5α-reduced androgens, dihydrotestosterone cannot serve as an aromatase substrate as A ring reduction prevents this (Larsen, 2002).

Daily production rates of DHT in men are between 280 μg (Mahoudeau et al., 1971) and 400 μg, with testosterone production being ~5 mg per day (Murray RK, 2006). Approximately 85% of DHT production is from testosterone, with the remainder from androstenedione (Mahoudeau et al., 1971), and the amount of testosterone available to serve as substrate is the key determinant of DHT production rates (Larsen, 2002). Sites of testosterone conversion to DHT mirror sites of 5αR expression (Table 1-1), with principal sites of DHT production being reproductive organs, skin and liver. There is a small amount of DHT produced in adipose (Longcope and Fineberg, 1985); in contrast, skeletal muscle can metabolise DHT, however is not considered a

significant site of its synthesis (Thigpen et al., 1993, Longcope and Fineberg, 1985) .

While its synthesis is irreversible, DHT metabolism is reversible, therefore actions of metabolising enzymes contribute to regulation of tissue exposure to DHT. As with other 5α-reduced steroids, DHT is metabolised by 3α- and 3β- HSDs, with subsequent formation of glucuronic acid conjugates prior to excretion in urine (Pirog and Collins, 1999, Belanger et al., 2003). 3αHSD reductase activity is approximately 5 times greater than 3βHSD reductase activity, both *in vitro* (Pirog and Collins, 1999) and *in vivo* (Mahoudeau et al., 1971), and forms the major pathway for DHT metabolism. In contrast to rat models, no significant differences attributable to age or sex have been observed in this ratio in human liver (Pirog and Collins, 1999). The importance of local DHT metabolism is highlighted in both normal and abnormal tissues. In genital skin from both males and females, DHT is synthesised (by 5αR from testosterone, and by 3αHSD from 3αDiol) at a significantly greater rate than non-genital skin, though its degradation is comparable (Morimoto et al., 1991). In pathological processes such as benign prostatic hyperplasia, an increase in prostatic DHT concentration does not just reflect an increase in 5αR activity, but also a corresponding decrease in DHT metabolism via 3αHSD, 3βHSD and 17βHSD (Isaacs et al., 1983). In contrast, an increase in DHT inactivation is seen in visceral (but not

subcutaneous) adipose in abdominally obese women, perhaps reflecting a compensatory phenomena whereby already metabolically compromised adipose is protected from exposure to excess androgens (Blouin et al., 2003). The final metabolites of DHT prior to excretion are glucuronic acid conjugates; while this stage of DHT metabolism is less well understood, levels of these metabolites (and thus activity of these enzymes) have been shown to correlate with metabolic risk factors and proposed as potential future markers of metabolic disease (Vandenput et al., 2007, Miljkovic et al., 2011).

The most well recognised pathway of DHT synthesis is via 5αR action on testosterone. However, there are also so-called "backdoor" pathways (i.e. pathways independent of DHEA, androstenedione and testosterone) to DHT synthesis, which are increasingly recognised, particularly in prostate cancer research, and are summarised in Figure 1.6. This pathway stems from the 3α, 5α- HSD metabolite of 17-hydroxyprogesterone which can be metabolised to androsterone, and in turn can be metabolised via the actions of 17βHSD and 3αHSD enzymes to form DHT. The significance of these pathways is twofold. Firstly, these pathways may provide an important route of androgen production in castration resistant prostate cancer. Secondly, there are potential differences in the way these "backdoor pathways" are targeted by 5αR inhibitor drugs (discussed further in Section 1.3.2); it has been

suggested, though not well clarified, that dutasteride (a dual 5αR isozyme inhibitor), but not finasteride (a 5αR2 inhibitor), inhibits the backdoor pathway (Mohler et al., 2011).

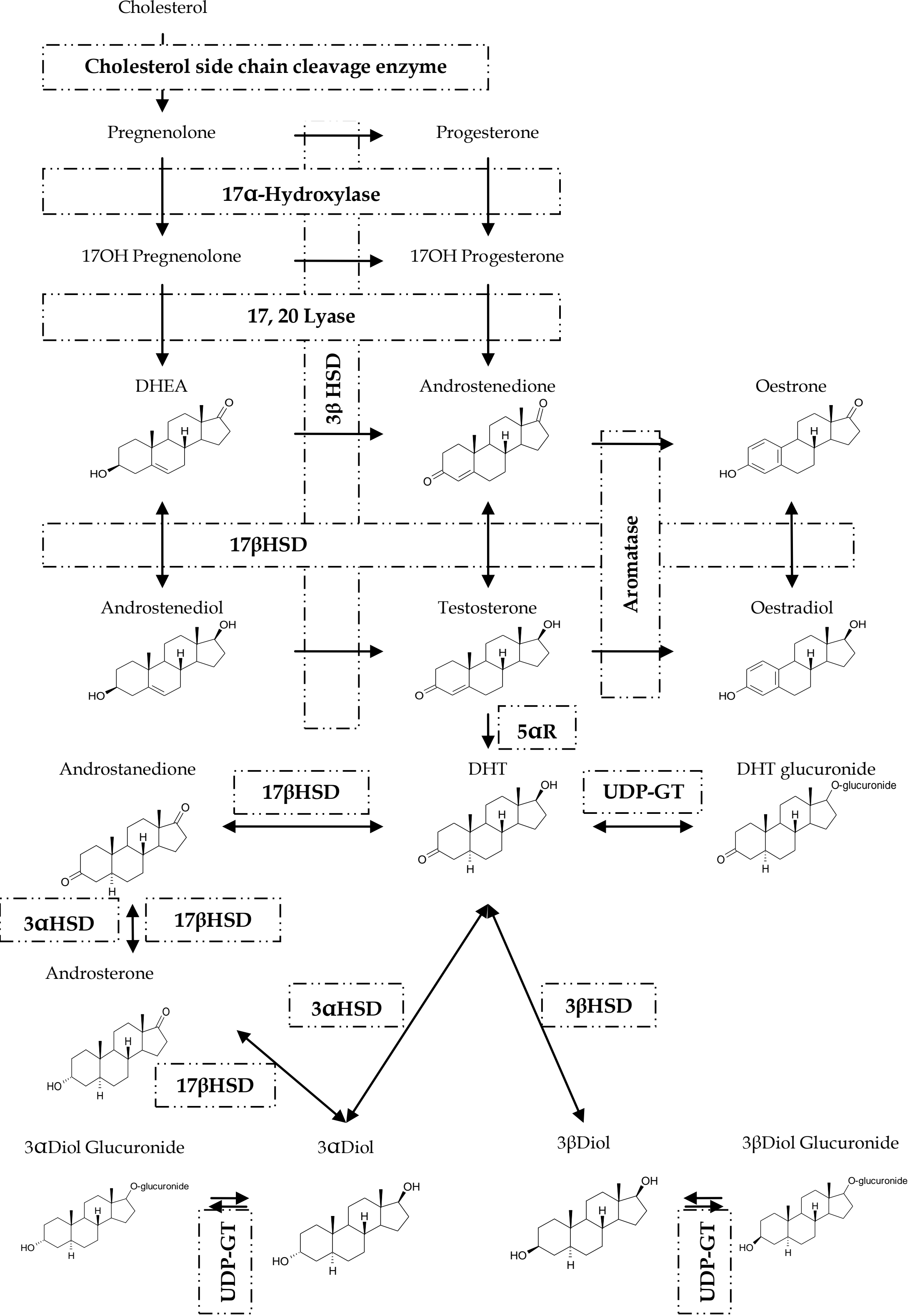

Figure 1.5 Principal pathways for synthesis and metabolism of testosterone and dihydrotestosterone. OH, hydroxy; HSD; hydroxysteroid dehydrogenase; DHEA, dehydroepiandrosterone; R, reductase; DHT, dihydrotestosterone; UDP-GT, Uridine diphosphate glucuronyl transferase (reverse reaction catalysed by β-glucuronidase); 3αDiol: 5α-androstan-3α, 17β-diol; 3βDiol: 5α-androstan-3β, 17β-diol.

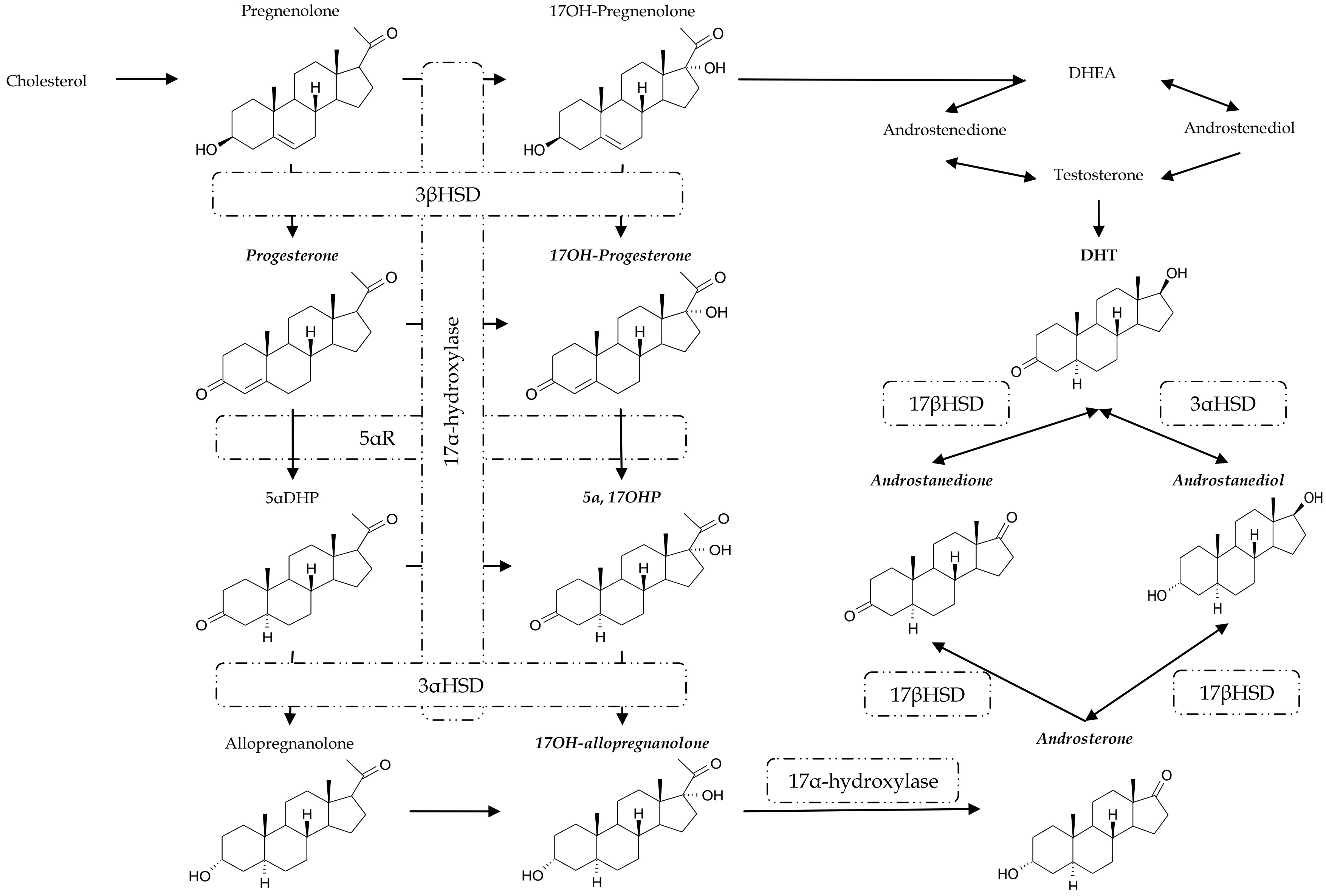

Figure 1.6 "Backdoor" pathway to dihydrotestosterone (DHT) synthesis. DHT can also be synthesised via pathways independent of dehydroepiandrosterone (DHEA), androstenedione and testosterone. Principal "backdoor pathway" intermediates are shown in italics, and enzymes catalysing formation of these intermediates are shown (boxed). OH, hydroxy; HSD, hydroxysteroid dehydrogenase; DH, dihydro; P, progesterone.

1.2.2 Mechanism of action

Androgens exert their effects through binding of the androgen receptor (AR); the two androgens able to bind with subsequent physiological effect are testosterone and dihydrotestosterone. AR is a member of the steroid hormone receptor family, with a structure highly conserved through evolution. It is encoded for on the X chromosome (Xq11-12), with eight exons; the first exon encodes the N-terminal domain, exons 2-3 the central DNA binding domain, and exons 4-8 encode the C-terminal ligand binding domain (Gelmann, 2002). AR is essentially ubiquitously expressed, with all tissues other than the spleen showing some AR expression (Gelmann, 2002).

Binding of AR triggers a chain of events similar to that described in detail later for glucocorticoids (Figure 1.8, Section 1.4.2), terminating in either activation or repression of gene expression. AR is located primarily in the cytoplasm (Bennett et al., 2010). Upon binding of testosterone or DHT to the cytoplasmic AR there is a release of heat shock proteins, conformational change exposing the activation function 2 (AF-2) binding surface and translocation to the nucleus. The AR-ligand complex can then bind specific androgen response elements on DNA (Gobinet et al., 2002), and either transactivation or transrepression of gene transcription then follow. DHT acts via the same pathway as testosterone, but does so with greater receptor affinity and subsequent effect (Bruchovsky and Wilson, 1968b, Askew et al.,

2007), with a dissociation constant (Kd) in prostate from AR of 3.4 x 10^{-10} M for dihydrotestosterone, compared with 0.49 nM for testosterone (Wilson and French, 1976).

Testosterone and its androgen metabolites do not solely bind AR. DHT and its 3β- reduced metabolite 5-androstane-3,17-diol have been shown to exert effects via the oestrogen β receptor in neuronal cell lines (Pak et al., 2007), and the latter can modulate the stress response and behavioural phenotypes in *in vitro* and *in vivo* models (Handa et al., 2008). Androgens are also ligands for other receptors including the steroid X receptor (Zhou et al., 2009), however all alternate receptor pathways are not as well understood as those mediated via the androgen receptor.

In addition to genomic mechanisms of action, androgens also have non-genomic signalling actions. The term 'non-genomic' describes modes of androgen action that are rapid (seconds to minutes), are membrane mediated, and do not involve (at least initially) transcription or translation (Foradori et al., 2008). These mechanisms may play a role in reproductive physiology and animal behaviour, however at this stage are not well understood (Foradori et al., 2008).

Non-classical pathways for androgen action can be demonstrated in animal models. An example is in the demonstration that testosterone exerts its effects on atherogenesis in both an AR dependent and independent manner; in an Apo-E deficient model of predisposition to atherosclerotic disease, AR knockout mice respond favourably to testosterone therapy, but to a lesser degree than their wild-type counterparts (Bourghardt et al., 2010).

Understanding androgen action is imperative for the understanding and treatment of androgen-dependent disorders. Disorders characterised by a decrease in androgen action (such as hypogonadism) can be treated with synthetic androgens. The long-term potential for adverse effects temper the use of androgen replacement therapies, and monitoring for occult prostate cancer is considered a mandatory aspect of therapeutic monitoring (Rhoden and Morgentaler, 2004), though in the absence of adequate trial data this has largely been recommended due to hypothetical risks and increased prostate cancer risk may not be seen in practice (Marks et al., 2006, Page et al., 2011b). In order to alleviate potential for long-term harm, the use of selective androgen receptor modulators (SARMs) are the subject of ongoing research, with the aim to give positive tissue-specific effects on muscle and bone, while sparing the prostate (Haendler and Cleve, 2012). The inability for the enzymes 5αR and aromatase to convert SARM molecules to their 5α-reduced and aromatised metabolites respectively likely affords them some of their

tissue specificity (Haendler and Cleve, 2012). Conditions where excess or abnormal androgen action are deleterious can be treated with anti-androgen therapies (usually used in advanced prostate cancer) or with drugs targeting androgen synthesis or metabolism, such as 5αR inhibitors (discussed in Section 1.3.2).

1.2.3 Androgens in health and disease

Androgens play an important role in health in both sexes, with the importance of androgens in male physiology being the focus of this section. Androgens are critical in pre-natal sexual differentiation (Cunha et al., 1992) and remain vitally important throughout post-natal life particularly in establishment of secondary sexual characteristics at puberty (Imperato-McGinley et al., 1979) and maintenance of this male phenotype thereafter. In addition, androgens play an essential part in spermatogenesis and fertility (Dohle et al., 2003).

In addition to the pivotal role played in sexual function and reproduction, androgens are also important in other organ systems. Androgens influence bone growth both directly as androgens, and indirectly following aromatisation and formation of oestrogens. Overall androgens preserve bone

density principally through maintenance of cancellous bone (Vanderschueren et al., 2004).

Body composition is influenced by androgens, principally testosterone. Muscle mass is increased by testosterone; a finding largely seen in studies of testosterone therapy in hypogonadal men where an increase in muscle mass is seen (Brodsky et al., 1996, Schroeder et al., 2005) resulting from an increase in muscle protein synthesis (Brodsky et al., 1996). Decreased testosterone is associated with increased fat mass (Mammi et al., 2012), and treatment with synthetic androgens decreases visceral adiposity (Wang et al., 2000, Schroeder et al., 2004) or prevents its accumulation (Allan et al., 2008).

Just as androgens are of such importance to health in all stages of life, aberrations in androgen physiology can have pathological consequences. In adulthood principal pathological processes include those where a decline in androgen concentrations is seen, such as hypogonadism, and those where excess androgen action is implicated in diseases such as prostate cancer, androgenic alopecia and benign prostatic hyperplasia.

1.3 Benign prostatic hyperplasia

1.3.1 Overview of benign prostatic hyperplasia

Benign prostatic hyperplasia (BPH) is a condition characterised by a non-cancerous increase in cell number within the prostate gland. As the prostate surrounds the urethra, clinically BPH manifests as a constellation of symptoms, often described by the umbrella term 'lower urinary tract symptoms' (LUTS), which can include urinary frequency, hesitancy, nocturia, and incomplete bladder emptying. BPH is the most common prostate condition, histologically present in approximately 50% of men at 50 years, rising to 80% at 80 years (Guess et al., 1990). On a population basis these figures are highly comparable to those who are symptomatic of BPH, though it has been consistently found that these findings are not necessarily true on an individual basis (Guess et al., 1990). Studies also report weak correlations between sonographically determined prostate transition zone volumes and reported symptoms (Franciosi et al., 2007). It follows, that the pathophysiology of benign prostatic hyperplasia is complex.

The prostate is a male exocrine gland composed of both glandular epithelium and stromal tissues, all contained within the prostatic capsule. Glandular epithelial cells produce important components of semen which aid in sperm motility and thus function. Stromal structures include sphincters, the

neurovascular bundle and prostatic capsule. Glandular regions of the prostate are anatomically divided into central, peripheral and transition zones. In the normal prostate the peripheral zone comprises 70% of glandular prostate mass, the central zone 25%, and the transition zone 5% (McNeal, 1988). The peripheral zone is affected by the vast majority of inflammatory and malignant disorders of the prostate. A smaller number occur in the transition zone, while the central zone is resistant to these conditions. In contrast, BPH affects primarily the transition zone, with some cases affecting the central peri-urethral zone (McNeal, 1988). Glandular architecture in BPH is normal.

Both epithelial and stromal tissues express the androgen receptor, and while the former directly mediates androgen effects on prostate growth, the latter does so indirectly through androgen stimulation of stromal growth factors which in turn stimulate prostatic epithelial proliferation (Schalken, 2005). Prostate size is determined through a complex and delicate balance of androgen-mediated growth and apoptosis (Isaacs, 1996, Mirone et al., 2006). There are important differences in the response of different prostatic cell types to androgens, whereby AR in epithelial cells functions to suppress growth, whereas in stromal cells functions to stimulate proliferation (Bauman et al., 2006, Niu et al., 2008a, Niu et al., 2008b); these regional

differences are important not only in health but also in understanding prostate disease and designing new therapeutic approaches.

While testosterone was previously considered the main 'male' sex hormone, the discovery that it is DHT that exerts intra-prostatic androgen effects provided a key breakthrough in androgen and prostate research (Marks et al., 2008). The growth of the prostate follows the trajectory of serum testosterone at puberty (Cunningham, 1996) and androgens are at least permissive players in prostatic growth. This understanding stems from the fact that prepubertal orchidectomy prevents prostate cancer, and orchidectomy is employed as an effective therapeutic modality in advanced prostate cancer (Isaacs, 1996). Despite the importance of androgens in prostatic growth, both testosterone and DHT concentrations decline with age, making it unlikely that BPH is solely an androgen-dependent disorder (Starka et al., 2009). It is also recognised however that circulating concentrations of androgens do not necessarily reflect the intra-prostatic environment (Marks et al., 2008). In BPH intra-prostatic DHT concentrations are not elevated, however this is the context of declining plasma testosterone levels with age, therefore may be considered to be relatively increased (Bartsch et al., 2002).

Whilst not negating the importance and direct influence of androgens on prostatic size and BPH, a broadening of research interests has been seen in recent years. In particular establishing other causative factors in BPH has been a key focus, with an aim to improving current therapeutic options. Prominent amongst recent research publications has been in establishing the link between BPH and the metabolic syndrome. While both BPH and the metabolic syndrome increase in prevalence as men age, an aetiological link between these two conditions was hypothesised following epidemiological studies. Multiple cohort studies have found associations with insulin resistance, obesity and low levels of physical activity with an increased incidence of BPH (Moul and McVary, 2010, Parsons et al., 2006), and increasing physical activity has been shown to reduce the risk of BPH (Parsons and Kashefi, 2008). The underlying mechanism for this observed association has not been established. Proposed theories include trophic effects of insulin in hyperinsulinaemic individuals (Hammarsten and Peeker, 2011), inflammation as suggested by both a significant increase in C-reactive protein levels seen amongst patients with lower urinary tract symptoms (Kupelian et al., 2009), and a weak but significant relationship between histological inflammation on prostate biopsy and lower urinary tract symptoms (Nickel et al., 2008). Serum concentrations of cortisol do not differ between BPH patients and age-matched controls (Tan et al., 2003). In response to a stressful task, measurement of salivary cortisol shows no

correlation with objective measures of BPH severity, however there is a significant correlation with subjective measures in terms of higher bother and impact scores (Ullrich et al., 2007). More detailed measures of the link between glucocorticoids and BPH have not been reported in published literature. Much remains to be understood regarding the aetiology of BPH, however aberrations in level and function of several hormones, particularly androgens and insulin, are likely to be contributing factors, and are the subject of ongoing research.

1.3.2 Treatment of benign prostatic hyperplasia, focusing on 5α-reductase inhibitors

Treatment modalities in BPH include observation, lifestyle modifications, medical or surgical therapy; the latter is usually reserved for those in whom medical therapy has failed. Medical treatments fall into two main categories, namely alpha adrenergic blockers and 5αR inhibitors. The aim is to improve symptoms and reduce complications such as urinary retention. Characteristics of commonly used medications are listed in Table 1-2. Monotherapy is generally commenced with an alpha blocker, which in the first year of treatment are more effective than 5αR inhibitors (Wilt and N'Dow, 2008). The mode of action of α blockers has traditionally been described as relaxation of prostate smooth muscle as α1 receptors are

abundant in prostate (Lepor, 2009), though this simplistic view is disputed (Lepor, 2007, Lepor, 2009). Alpha 1 (α1) receptors are widely distributed throughout the body, and are currently categorised into three subtypes – $\alpha 1_A$, $\alpha 1_B$, and $\alpha 1_D$ (2009). Tamsulosin is selective for $\alpha 1_A$ and $\alpha 1_D$ receptors, with 10 fold greater affinity than for $\alpha 1_B$, though this affords it no additional clinical benefit (Lepor, 2007). The main reason why tamsulosin is often selected over other α blockers is that no dose titration is needed due to lack of a significant first-dose hypotensive effect.

Many compounds, broadly classified as steroidal and non-steroidal drugs, have been investigated as potential 5αR inhibitors, and have been comprehensively reviewed (Aggarwal et al., 2010). The only two 5αR inhibitors licensed for use in humans are finasteride and dutasteride; both steroidal compounds. Finasteride was licensed for use in BPH in 1992, and dutasteride followed a decade later in 2002. The main difference between these two drugs can be expressed as their *in vitro* potency against 5αR, measured as the amount of drug required to inhibit enzyme action by 50% (half maximal inhibitory concentration, IC_{50}). Against human 5αR2, finasteride and dutasteride have IC_{50} values of <0.1 nM and 0.5 nM respectively. In contrast, a marked difference between these compounds is seen in their activity against human 5αR1, where finasteride has an IC_{50} of 52 nM, compared with the more potent dutasteride with an IC_{50} of 2.4 nM

(Aggarwal et al., 2010). At concentrations used *in vivo*, finasteride in humans acts to inhibit 5αR2 alone (Tian, 1996), compared with dutasteride which acts to inhibit both 5αR1 and 5αR2 (Frye, 2006).

Another important consideration with 5αR inhibitor drugs is their selectivity towards 5αR; the main consideration being that they do not cause a decrease in synthesis of 5αR substrates, nor inhibit other enzymes metabolising 5αR substrates, particularly 5βR. Several 3-oxo-4-azasteroid compounds tested have been shown to have significant inhibitory activity against 3βHSD (Brandt and Levy, 1989); the exact mechanism of this inhibition has not been established. However, at clinically relevant doses, there is no significant inhibition of 3βHSD by finasteride (Brandt and Levy, 1989) or dutasteride (Frye, 2006). While known to be a competitive inhibitor, at clinical doses there is not thought to be effective inhibition of 5βR by finasteride (Drury et al., 2009). The inhibitory activity of dutasteride against 5βR is not described. Dutasteride inhibits 5αR3, while finasteride is not thought to (Mohler et al., 2011), however consequences of drug inhibition of 5αR3 are not well understood.

After three months of treatment, dutasteride leads to a >90% reduction in both serum and intra-prostatic DHT concentrations (Wurzel et al., 2007). This is associated with an increase in testosterone, however these testosterone

concentrations are not significantly different from concentrations in placebo-treated men. Finasteride also lowers serum DHT , though to a lesser degree at approximately 70% suppression (Andriole and Kirby, 2003, Clark et al., 2004). Both have demonstrated effect in those with moderate to severe disease (Gravas and Oelke, 2010), and while dutasteride lowers circulating and prostatic DHT concentrations to a greater degree, this has not been shown to result in clinically significant differences in efficacy (Andriole and Kirby, 2003, Marihart et al., 2005). The evidence behind the efficacy of dual therapy (i.e. α blocker + 5αR inhibitor) suggests an overall benefit (Montorsi et al., 2010), though findings are not consistent (Gravas and Oelke, 2010) and practice does vary between centres. Improvements in longterm outcomes with dutasteride therapy may not be in BPH itself, but may be due to a hypothesised reduction in prostate cancer risk (Goldenberg et al., 2009), however the role of 5αR inhibitors in prostate cancer is still unclear (Nacusi and Tindall, 2011) and at this stage are not used in this context outside of clinical trial settings.

5αR inhibitors are not just utilised in treatment of prostate disease. Finasteride is used in androgenic alopecia treatment with moderate evidence for clinical efficacy (Mella et al., 2010), though in this context is given at a lower dose (1 mg daily) than is used in BPH. In women with hirsutism, finasteride is utilised as an anti-androgen (Escobar-Morreale et al., 2012), and

is effective in doses of 2.5 - 5 mg daily (Bayram et al., 2002). Although 5αR1 is highly expressed in skin and hair follicles (Eicheler et al., 1995), dutasteride is not licensed for treatment of alopecia or hirsutism.

	Tamsulosin	Dutasteride	Finasteride
Structure			
Drug Class	α Blocker	5αR Inhibitor	5αR Inhibitor
Target	$\alpha 1_A$ and $\alpha 1_D$ receptors	5αR1 and 5αR2	5αR2
Usual dose (BPH)	0.4 mg po daily	0.5 mg po daily	5 mg po daily
Bioavailability	>90%	60%	80%
T_{max}	4-5 hours	2-3 hours	1-2 hours
$T_{1/2}$	14-15 hours (modified release formulation)	3-5 weeks	6-8 hours
Protein binding	94-99%	99.5%	90%
Serum DHT suppression	Unknown	94.7%	70.8%
Prostate DHT suppression	Unknown	93-95%	80-90%
Metabolism	Hepatic: CYP3A4 and CYP2D6	Hepatic: CYP3A4	Hepatic: CYP3A4

Table 1-2 Drug Characteristics. T_{max}, time to maximum concentration; $T_{1/2}$, elimination half life; BPH, benign prostatic hyperplasia; DHT, dihydrotestosterone; 5αR, 5α-reductase; po, oral administration Sources: Finasteride and dutasteride (Gravas and Oelke, 2010, Marks et al., 2008); tamsulosin [Merck drug information sheet].

While 5αR inhibitors are clinically used for their anti-androgen properties, they could also have clinically relevant effects on other substrates of 5αR. This thesis will focus on the metabolic and hypothalamic-pituitary-adrenal axis effects of 5αR inhibition, which are not only be influenced by modulation of androgen physiology, but also by alterations in glucocorticoid physiology.

1.4 Glucocorticoids

1.4.1 Significance, synthesis and metabolism

Glucocorticoids are steroid hormones produced by the zona fasiculata of the adrenal cortex under control of the hypothalamic-pituitary-adrenal (HPA) axis. Corticotrophin releasing hormone (CRH) produced by the hypothalamus stimulates the anterior pituitary to produce and secrete adrenocorticotrophic hormone (ACTH). ACTH in turn stimulates adrenal glucocorticoid synthesis and cortisol acts to negatively feedback and regulate its own production. Glucocorticoid effects are predominantly initiated by binding to the intra-cellular glucocorticoid receptor (GR), with the resultant cascade influencing gene expression. Glucocorticoids play a key role in many essential biological processes, whether for basal homeostasis or stress-related responses. These actions are varied and involve growth, development,

regulation of glucose metabolism, and responses to stress, infection, and inflammation.

The quantitatively most abundant and physiologically most relevant endogenous glucocorticoids are considered to be cortisol and corticosterone. Cortisol is the main glucocorticoid in humans, accounting for approximately 30% of adrenocortical hormone synthesis (Barrett KE, 2010), and circulating concentrations of cortisol are 10-20 times greater than those of corticosterone (Hariharan et al., 1992), largely a reflection of the fact that production rates of corticosterone are approximately one third that of cortisol (Barrett KE, 2010). Rodents lack adrenal 17α-hydroxylase, and thus utilise corticosterone as their main glucocorticoid (Laplante et al., 1964).

Variations in glucocorticoid concentration or action influence many, and cause some, pathological processes. Glucocorticoid excess is seen clinically in Cushing's syndrome, with multi-system consequences including insulin resistance, obesity, dyslipidaemia and depression. Glucocorticoid deficiency, as seen in Addison's disease, can be potentially fatal in the face of injury or illness that would typically be overcome via a response mounted by endogenous glucocorticoids. It is not only aberrant glucocorticoid production that can manifest clinically, but also pathological entities involving impaired glucocorticoid action. Glucocorticoid resistance is associated with a range of

underlying abnormalities including GR receptor mutations, defective GR binding, translocation or transactivation (processes described in 1.4.2) (Barnes, 2010). Clinically this can manifest as patients with poor glucocorticoid response in usually glucocorticoid responsive disease states such as asthma or rheumatoid arthritis.

Control of both circulating and intracellular cortisol concentrations are vital to health. Intracellular regulation of glucocorticoids is influenced by the activity of glucocorticoid regenerating and degrading enzymes. While the contribution of the adrenals to cortisol synthesis is vital, several studies have demonstrated the considerable importance of tissue glucocorticoid production (Andrew et al., 2002, Stimson et al., 2009, Basu et al., 2004). Glucocorticoid metabolism within tissues occurs reversibly via the 11β-hydroxysteroid dehydrogenases (11βHSD), and irreversibly via the 5α- and 5β-reductases (Figure 1.7). The 11βHSD enzymes catalyse the reaction between active cortisol with inactive cortisone, corresponding to a biologically significant structural change of a carbon 11 hydroxyl to keto group. 11βHSD has two known isozymes. 11βHSD1 acts predominantly as a reductase *in vivo*, catalysing the formation of cortisol from cortisone. 11βHSD2 acts in the opposite direction and inactivates cortisol.

Morbidity is associated with abnormal glucocorticoid metabolism. In the rare autosomal recessive disorder Syndrome of Apparent Mineralocorticoid Excess (SAME), a lack of 11βHSD2 means cortisol is not inactivated in mineralocorticoid target tissues, and is therefore able to act as a mineralocorticoid receptor agonist with resulting hypertension and hypokalaemia (Wilson et al., 2001). Research characterising the role of 11βHSD1 in glucocorticoid regulation perhaps best exemplifies the ability of tissue glucocorticoids to affect metabolism without altering circulating cortisol concentrations. In the context of normal or near-normal plasma cortisol concentrations in metabolic disease, tissue-specific dysregulation of cortisol regeneration by 11βHSD1 has been demonstrated in adipose (Rask et al., 2002) and liver (Baudrand et al., 2010, Baudrand et al., 2011). Importantly, alterations in tissue glucocorticoid concentrations have been shown to affect insulin sensitivity (Masuzaki et al., 2001, Morton et al., 2001). The pharmacological inhibition of 11βHSD1 remains an area of intense research interest with an attempt to develop inhibitors selective to only the detrimental effects of elevated intra-tissue glucocorticoid effects (Hughes et al., 2008, Morton, 2010).

Enzymatic regulation of cortisol metabolism also impacts on circulating cortisol concentrations. Enhanced peripheral glucocorticoid clearance results in less negative feedback inhibition of ACTH-dependent steroids. This is

seen in conditions such as PCOS (Rodin et al., 1994) and possibly during growth hormone treatment (Isidori et al., 2003) where enhanced cortisol clearance, associated with decreased 11βHSD1 activity, is thought to contribute to activation of the HPA axis with (as a byproduct of attempts to increase cortisol secretion) an increase in adrenal androgens (Rodin et al., 1994). These features are particularly apparent in the rare Apparent Cortisone Reductase Deficiency (Jamieson et al., 1999). While targeting metabolic disease, the potential for activation of the HPA axis in response to reduced negative feedback following 11βHSD1 inhibition is the subject of ongoing research (Harno, 2010). Conversely, decreased peripheral glucocorticoid clearance is also associated with pathology; with decreased activation of the HPA axis from reduced peripheral clearance potentially rendering individuals less able to adapt to stressful stimuli (Yehuda and Seckl, 2011).

The influence exerted by 11βHSDs demonstrates the importance of enzymatic regulation in glucocorticoid physiology. Additional enzymatic control of glucocorticoids is in the initial, rate-limiting, step in glucocorticoid metabolism: the reduction of the carbon 4-5 double bond by 5αR or 5βR. The action of 5αR and 5βR in inactivating cortisol occurs principally in the liver (Gold, 1961). The formation of dihydrocortisol (DHF) or dihydrocortisone (DHE) is followed by further reduction to tetrahydrocortisol (THF) or

tetrahydrocortisone (THE) respectively. These are then conjugated and excreted mostly in urine with around 15% excreted in faeces (Barrett KE, 2010). Approximately 50% of secreted cortisol is excreted as βTHF, αTHF and THE, 25% as cortols/cortolones, 10% as C19 steroids, and 10% as cortolic/cortolonic acids, with the remaining being free unconjugated steroids (Tomlinson et al., 2004). Alternate pathways for excretion exist, with either 6β-hydroxylation, or (for 10% of secreted cortisol and cortisone) conversion to 17-ketosteroid or 20-hydroxy derivatives which are then conjugated and excreted in urine (Barrett KE, 2010).

The irreversible reduction of the carbon 4-5 double bond can be catalysed by either 5α- or 5β-reductase; normally 5α:5β metabolites are formed in a 1:2 ratio (Tomlinson et al., 2004), however this ratio is altered in several disease states. It is significantly higher in hyperthyroidism, significantly lower in hypothyroidism (Hoshiro et al., 2006), and in both cases this is due to differences in 5αR rather than 5βR activity. As described above, the aetiology of PCOS may be related to increased glucocorticoid metabolism, with a subsequent decrease in negative feedback causing increased ACTH stimulation of adrenal androgens (Stewart et al., 1990, Tsilchorozidou et al., 2003). In lean women with PCOS, there is a significant increase in 5αR activity, with the 5αTHF:5βTHF ratio rising to approximately 0.7 (Tsilchorozidou et al., 2003). This finding has been reiterated in a study

showing increased 5αR activity in both obese and non-obese PCOS patients (Vassiliadi et al., 2009). The pathophysiology of obesity and fatty liver is still unclear, but altered glucocorticoid metabolism is thought to play a significant role (Roberge et al., 2007). In obese Zucker rats, an increase in hepatic 5αR activity was seen, with a much greater proportion of 5α:5β corticosterone metabolites formed (Livingstone et al., 2000a). In humans, obesity and female gender are associated with increased 5αR activity (Andrew et al., 1998, Fraser et al., 1999, Reynolds et al., 2001), and in men with fatty liver an increase in 5βR is seen (Westerbacka et al., 2003).

5αR transcript abundance in adipose is lower in women than men, however does not correlate with either generalised or central obesity (Wake et al., 2007). A positive correlation has been demonstrated between hepatic 5αR mRNA expression and hyperinsulinaemia in obese patients – hypothesised to be an adaptive response aiming to preserve hepatic insulin sensitivity in the face of raised glucocorticoid concentrations (Baudrand et al., 2011). In two very rare cases of 5αR deficiency, a lower 5α-reduction of cortisol (as well as testosterone) is reported (Fisher et al., 1978), however clinical consequences of this were not described.

Alterations in 5αR activity are studied in animal models of human disease, where variations in activity are also seen. Hepatic 5α-reduction of

corticosterone in rats is increased in obesity (Livingstone et al., 2000a, Livingstone et al., 2005) and obesity is, at least partially, related to glucocorticoid concentrations as adrenalectomy attenuates weight gain, and normalises omental fat 11βHSD1 activity (Livingstone et al., 2000b). This increased 5αR activity is thought to be independent of androgens, as gonadectomy does not alter the relative difference in 5αR expression between lean and obese animals (Barat et al., 2007).

It is not only the substrates of 5αR that have biological relevance; metabolites 5αDHB and 5αTHB can also bind GR (McInnes et al., 2004). Upon binding GR, 5αTHB can activate mRNA expression from glucocorticoid responsive genes, and contribute to central negative feedback (McInnes et al., 2004). 5αTHB also displays both *in vitro* and *in vivo* anti-inflammatory effects, without metabolic toxicity (Yang et al., 2011). 5αDHB does not have any mineralocorticoid activity (Sekihara et al., 1978), however the mineralocorticoid activity of 5αTHB is unknown. 5αDHB binding to the SXR has been demonstrated (Blumberg et al., 1998), though it remains unknown how physiologically relevant this pathway is.

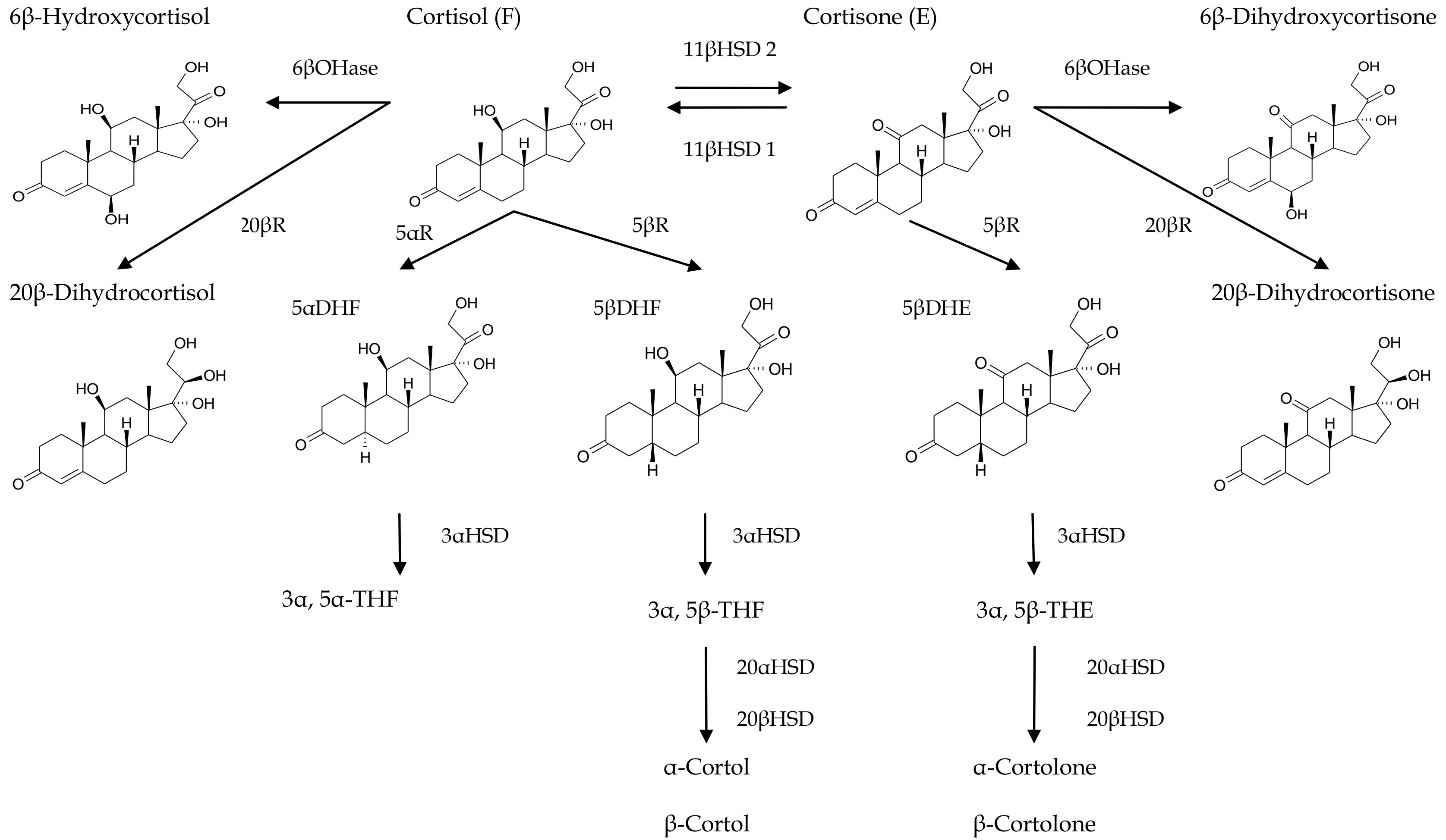

Figure 1.7: Principal pathways of glucocorticoid metabolism. OHase, hydroxylase; HSD, hydroxysteroid dehydrogenase; R, reductase; DHF, dihydrocortisol; DHE, dihydrocortisone; THF, tetrahydrocortisol; THE, tetrahydrocortisone. Metabolism of 5α- and 5β- metabolites also occurs via 3βHSD.

1.4.2 Mechanisms of glucocorticoid action

Following adrenocortical synthesis, glucocorticoids are released into the systemic circulation. Corticosteroid-binding globulin (CBG) binds the majority of glucocorticoids, rendering them biologically unavailable. Albumin also binds glucocorticoids with low affinity but high capacity (van der Laan and Meijer, 2008). Lipophilic in nature, glucocorticoid molecules are thought to diffuse through the cell membrane (Zhou and Cidlowski, 2005), however there is evidence of steroid transporters such as the multidrug resistant P-glycoprotein at the blood-brain barrier (Karssen et al., 2001), the organic anion transporter 3 in adrenocortical cells (Asif et al., 2005), and the glucocorticoid importer demonstrated in rat liver (Lackner et al., 1998). Once inside the cell, glucocorticoids bind their intra-cellular receptors, including GR. With such a wide array of glucocorticoid actions, it is not surprising that the glucocorticoid receptor is essentially ubiquitously expressed.

The human glucocorticoid receptor (hGR) gene comprises 9 exons and is situated on chromosome 5. Two homologous receptor isoforms, designated α and β, occur as a result of exon 9 splicing (Nicolaides et al., 2010). There is further complexity in GR function due to translation of both α and β transcripts into two distinct proteins, named hGR-A and hGR-B, with the

latter demonstrating greater efficacy in transactivation, though similar in other respects (Zhou and Cidlowski, 2005). In addition, hGR is subject to post-translational modification, though the physiological relevance of this is not well understood (Zhou and Cidlowski, 2005). hGRβ isoform exists in human and zebrafish, though was traditionally thought not to be present in rodents (Kino et al., 2009). The presence of GRβ mRNA and protein in murine cells and tissues has been shown, with reportedly similar properties to hGRβ (Hinds et al., 2010). hGR isoforms are structurally identical until amino acid 727, after which hGRα has 50, and hGRβ 15, distinct amino acids (Nicolaides et al., 2010). These structural differences correspond to a unique ligand-binding domain; with N-terminal domain and DNA binding domain preserved between isoforms (Kino et al., 2009). α and β isoforms of hGR have distinct functional properties. hGRα undergoes the classical conformational changes described below, is bound by glucocorticoids (both natural and synthetic) and influences gene transcription via binding to glucocorticoid response elements (GRE) (Nicolaides et al., 2010). In contrast, hGRβ does not bind glucocorticoid agonists (as its structural configuration renders the formation of a ligand binding pocket impossible), but rather exerts a dominant negative effect on the transcriptional activity of hGRα (Kino et al., 2009, Lewis-Tuffin and Cidlowski, 2006). hGRβ can however bind the ligand RU486, a glucocorticoid antagonist, which results in cytoplasm to nucleus translocation, and both up and down-regulation of

gene expression (Lewis-Tuffin et al., 2007); though the physiological relevance of this is yet to be clarified. hGRβ has been implicated in glucocorticoid-resistant rheumatoid arthritis where its expression is increased (Kozaci et al., 2007), as well as other inflammatory diseases where there is an enhancement of its dominant negative inhibition of hGRα (Barnes, 2010, Lewis-Tuffin and Cidlowski, 2006). However, the exact nature and importance of hGRβ is still unclear. Given that hGRβ cannot bind GR agonists, it is presumed, though not confirmed, to lack a direct role in the function of glucocorticoids and their metabolites.

Prior to ligand binding GR resides as part of a cytoplasmic polyprotein complex. GR is a ligand dependent transcription factor, and remains inactive in its unbound state. Ligand binding leads to conformational changes, unravelling of the polyprotein complex, and exposure of nuclear localisation signals. Upon glucocorticoid binding, GR is transported into the nucleus and, following homodimerisation, binds to glucocorticoid response elements (GREs) on target genes (Zhou and Cidlowski, 2005). In addition to homodimers, glucocorticoid action can also be mediated via monomers (Schacke et al., 2002) and multimers (Adams et al., 2003). The key glucocorticoid actions are categorised as transactivation and transrepression. Transactivation leads to activation of gene expression, as seen in the activation of tyrosine aminotransferase and phosphoenolpyruvate

carboxykinase (PEPCK), both enzymes involved in gluconeogenesis (Schacke et al., 2002). Transrepression is the inhibition of a transcription factor responsible for up-regulation of gene expression, with the net effect being down-regulation of gene expression. This can either occur via GR binding to negative GREs or through interaction with further transcription factors. Binding to negative GREs is seen in osteoblasts with resulting decrease in transcription of the bone protein osteocalcin (Stromstedt et al., 1991); which contributes to some of the long-term osteopenic consequences of glucocorticoid therapy (Schacke et al., 2002). Repression of pro-opiomelanocortion (POMC) gene transcription by glucocorticoids is also mediated via a negative GRE (Drouin et al., 1989). Transrepression without GR binding to DNA is seen when GR binds to a transcription factor that usually induces transcription of pro-inflammatory proteins. NF-κB and activating protein (AP)-1 are examples of proteins whose actions are inhibited when bound by GR (De Bosscher and Haegeman, 2009) (Figure 1.8).

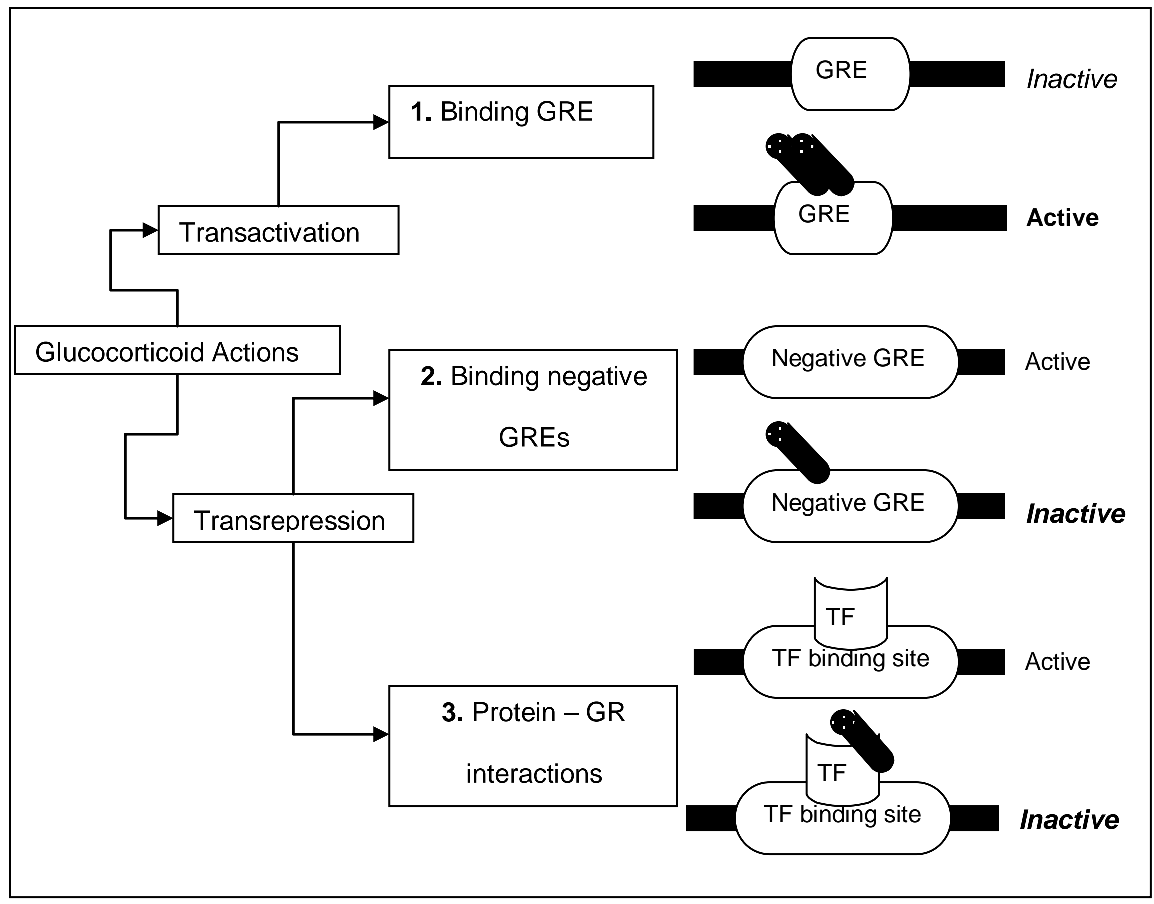

Figure 1.8: Mechanisms of glucocorticoid actions

Glucocorticoids (spotted circle) when bound to the glucocorticoid receptor (GR-solid black) alter gene transcription through three main pathways (1, 2 or 3): (adapted from (Schacke et al., 2002)) 1. **Transactivation involves binding of the homodimer to the glucocorticoid response element (GRE) on a target gene and activation of gene transcription.** 2. **Transrepression occurs via binding of a monomer to a negative GRE and repressing gene transcription.** 3. **Repression of gene transcription can also occur via the interaction of a monomeric ligand bound GR with a transcription factor (TF), which then inhibits the activation of gene transcription by these transcription factors.**

Although many of the biological properties of glucocorticoids are mediated via GR, at least two other classes of receptor bind with glucocorticoids, namely the mineralocorticoid receptor (MR) and the steroid and xenobiotic receptor (SXR). The latter is synonymous with the pregnane activated receptor (PAR) and in rodents the pregnane X receptor (PXR).

The mineralocorticoid receptor was cloned in 1987 by Arriza and colleagues (Arriza et al., 1987), revealing a 984 amino acid protein encoded on chromosome 4. When comparing human MR with human GR, there is 94% homology with the DNA binding domain and 57% with the ligand binding domain. In terms of ligand affinity, the mineralocorticoid receptor is certainly not exclusively bound by mineralocorticoids. The affinities for steroidal ligands are approximately deoxycorticosterone = corticosterone ≥ aldosterone = cortisol (Funder, 2005) and circulating concentrations of glucocorticoids are at least 100-1000 times greater than circulating aldosterone (Yang and Young, 2009). The fact that aldosterone is able to exert any physiological effect at all is largely explained by the co-expression of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) in MR expressing cells (Edwards et al., 1988, Funder et al., 1988). 11βHSD2 converts cortisol into receptor-inactive cortisone, thereby preventing MR activation by glucocorticoids. Myocardium and hippocampus are amongst the most notable tissues lacking significant 11βHSD2 activity, where cortisol (or

corticosterone) is the most significant MR ligand rather than aldosterone (Yang and Young, 2009). It is worth noting that while glucocorticoids bind MR, mineralocorticoids such as aldosterone are not known to bind GR (Yang and Young, 2009).

SXR is a nuclear receptor most prominently expressed in liver and intestine, which plays a key role in transcriptional regulation of *CYP3A4* (Zhou et al., 2009). SXR has a wide range of ligands ranging from glucocorticoids to drugs such as the antituberculous drug rifampicin. While traditionally considered an 'orphan' receptor, increasing bodies of evidence suggest a greater role for SXR in endogenous glucocorticoid metabolism than previously thought. *In vitro* studies demonstrate dexamethasone to be a weak activator of human and mouse SXR (Moore et al., 2000). More convincing evidence still comes via gene knockout models, where traditional inducers such as dexamethasone fail to cause *Cyp3a4* gene activation (Zhou et al., 2009). The role of SXR in glucocorticoid and mineralocorticoid homeostasis in transgenic mice was investigated by Zhai et.al. (Zhai et al., 2007), showing activation of SXR causes an increase in corticosterone concentrations, and an increase in adrenal enzymes involving in steroid synthesis. These alterations happen independently of the HPA axis, so that hypercortisolism co-exists with a normal response to a dexamethasone suppression test.

In addition to receptor mediated effects, glucocorticoids also act via non-genomic routes. These actions are rapid and do not directly involve effects on gene transcription classically associated with glucocorticoid activity. Non-genomic effects of glucocorticoids are widespread and are now considered important mediators of effect for both endogenous and synthetic glucocorticoids (Borski, 2000, Stahn et al., 2007).

1.4.3 Glucocorticoids in health

Glucocorticoids are vital to many aspects of health, particularly immunity and macronutrient metabolism. In essence, glucocorticoids act to promote synthesis and mobilisation of energy substrates such as glucose and fatty acids, and are particularly important in times of stress, inflammation and infection.

Glucocorticoids influence glucose homeostasis in a counter-regulatory manner to insulin; they increase plasma glucose concentrations by increasing gluconeogenesis and decreasing peripheral glucose uptake. Glucocorticoids stimulate gluconeogenesis by increasing gene expression of key enzymes involved in glucose production, including tyrosine aminotransferase (TAT) (Jantzen et al., 1987), phosphoenolpyruvate carboxykinase (PEPCK) (Hanson and Reshef, 1997) and glucose-6-phosphatase (Van Schaftingen and Gerin,

2002). In skeletal muscle glucose uptake is decreased, glycogen synthesis reduced and proteolysis induced to mobilise amino acids for use as gluconeogenic substrates (Vegiopoulos and Herzig, 2007). Effects of glucocorticoids on adipose tissue are complex and not completely understood, though they are known to exert effects which likely differ by time, adipose depot and prandial state (and corresponding changes in insulin and catecholamine concentrations), with an overall effect of increasing lipolysis when energy substrates are needed and promoting lipid storage when insulin concentrations are elevated (Macfarlane et al., 2008, Coppack et al., 1994, Lundgren et al., 2004). Effects of glucocorticoids on glycogen turnover are more akin to effects mediated by insulin, whereby both synthesis and lysis are promoted, perhaps in preparation for glucose release (Andrews and Walker, 1999, Macfarlane et al., 2008).

Both acute and chronic blood pressure regulation is influenced by glucocorticoids. While co-expression of 11βHSD2 in sites of MR expression such as kidney prevent excessive MR-mediated glucocorticoid activity (Edwards et al., 1988), glucocorticoids are able to increase blood pressure by enhancing angiotensin II-mediated effects and promoting sodium retention (Brem, 2001). Peripherally, glucocorticoids increase vascular tone both by potentiating the effects of vasoconstrictors such as catecholamines, and via direct effects on vascular smooth muscle cells increasing vascular tone and

thus peripheral vascular resistance (Ullian, 1999). While the role of glucocorticoids in a normotensive population has not been clearly demonstrated (Fraser et al., 1999), importance in hypertension, and in those at risk of hypertension, has been shown in terms of increased cortisol secretion and increased dermal sensitivity to synthetic glucocorticoids (Walker et al., 1998).

As key modulators of the immune system, glucocorticoids are vital in response to infection and inflammation, and act to inhibit production of pro-inflammatory molecules, influence recruitment of cells involved in the immune response, and induce transcription of anti-inflammatory genes (McEwen et al., 1997). Glucocorticoids also modulate bone turnover through a number of mechanisms (Schacke et al., 2002) resulting in decreased osteoblast and increased osteoclast activity, and circadian cortisol variation is reponsible for circadian variations seen in osteocalcin, a marker of osteoblast activity (Heshmati et al., 1998).

1.4.4 Glucocorticoids in disease and therapeutics

Aberrations in glucocorticoid concentrations and metabolism are detrimental to health, and these effects are principally seen as an impaired response to

stress and infection, increased fracture risk and increased metabolic and cardiovascular risk; the latter forming the focus of discussion in this section.

Addison's disease, a condition characterised by insufficient production of adrenal steroids, including glucocorticoids, is associated with an increased risk of cardiovascular disease (Bergthorsdottir et al., 2006). In addition, those with excess circulating glucocorticoid concentrations, such as patients with Cushing's syndrome, there is an increase in both cardiovascular morbidity and mortality when compared with the general population (Etxabe and Vazquez, 1994, Mancini et al., 2004). These risks are largely reversible upon treatment, however persistence of increased risk is seen long-term, particularly in those with pituitary Cushing's syndrome (Cushing's disease) (Colao et al., 1999, Giordano et al., 2011, Graversen et al., 2012). Common features between the adverse metabolic phenotype seen in Cushing's syndrome and those seen in the metabolic syndrome have led to the hypothesis that excess glucocorticoids may play a key role in the pathogenesis of the metabolic syndrome, and may therefore also provide a therapeutic target (Walker, 2006). In particular, there is an activation of the HPA axis seen in patients with the metabolic syndrome, of unconfirmed and likely multi-factorial aetiology, including effects of early life programming, chronic stress and abnormal tissue cortisol metabolism (Anagnostis et al., 2009).

The metabolic syndrome, comprised of obesity (particularly abdominal), hypertension, diabetes and dyslipidaemia, is the cluster of disorders most influential in the development and progression of cardiovascular diseases. The term is synonymous with the previously entitled 'syndrome X' or 'metabolic syndrome X'. The concept of the metabolic syndrome arose in the late 1980s (Reaven, 1988) with the observation that insulin resistance was associated, perhaps causally, with this constellation of diagnoses. Insulin resistance was characterised as a decrease in insulin-mediated glucose uptake, and reduction in insulin suppression of free fatty acid release from adipose, with resulting hyperglycaemia and hyperinsulinaemia.

Definitions of the metabolic syndrome have evolved over the years and do still vary by country and professional body. The current definition adopted by the Scottish Intercollegiate Guidelines Network (SIGN, 2007) is any three of the following:

- Increased waist circumference (≥102 cm in men, ≥88 cm in women; or ≥90 cm in Asian men, ≥80 cm in Asian women)
- Elevated triglyceride (≥1.7 mmol/L)
- Decreased high density lipoprotein (HDL) cholesterol (<1.03 mmol/L in men, <1.29 mmol/L in women)
- Blood pressure >130/85 mmHg or active treatment for hypertension
- Fasting glucose >5.6 mmol/L or active treatment for hyperglycaemia

Other features of the metabolic syndrome can include increased levels of small and atherogenic low density lipoprotein (LDL) molecules. In addition, there are many other chronic diseases which have been associated with the metabolic syndrome, including obstructive sleep apnoea and benign prostatic hyperplasia, though these are currently defined as parallel diagnoses. There is no specific treatment for the metabolic syndrome *per se*, rather the therapeutic approach lies in optimising lifestyle, promoting weight loss, and management of hypertension and diabetes as appropriate (SIGN, 2007).

Multiple studies demonstrate that each component of the metabolic syndrome has been linked with increased risk of cardiovascular complications, including abdominal obesity (Ibrahim, 2010), hypertension (Kannel, 1996), and dyslipidaemia (Catapano et al., 2011). Fasting glucose and simple measures of impaired glucose tolerance are useful measures on a population basis where time and resources are limited, though more detailed, sensitive and precise measures are more appropriate in a research context (Antuna-Puente et al., 2011). The concept of the metabolic syndrome is attractive in the holistic nature of its definition, however this approach has been criticised as it arguably does not add to strength of risk prediction that is afforded by viewing risk factors in isolation (Sattar et al., 2008), albeit most

studies do show a significant increase in cardiovascular risk associated with the metabolic syndrome (Cornier et al., 2008).

While insulin resistance appears to be at the heart of the pathophysiology of the metabolic syndrome (and its individual components), its underlying cause remains unknown. The role of aberrant intra-tissue metabolism of other hormones including glucocorticoids in inducing or worsening insulin resistance is a key research focus.

Additional evidence for the potential role of glucocorticoids in the metabolic syndrome is seen in the side effect profile of synthetic glucocorticoids. Therapeutics in a number of disease processes have utilised synthetic glucocorticoids for over half a century. Indications for glucocorticoid use vary greatly, and include asthma, inflammatory bowel disease, rheumatoid arthritis, peritumour oedema and prevention of transplant rejection. Dose, duration of therapy and route of administration are all adapted to clinical indication and patient characteristics. While being very effective, glucocorticoid therapy is perennially plagued by the relatively high risk of adverse effects, often in vulnerable patient populations.

In a dose-dependent manner, glucocorticoid therapy induces insulin resistance, with increased glucose and insulin concentrations, increased

proteolysis and decreased insulin-mediated peripheral glucose uptake and lipolysis suppression (van Raalte et al., 2011). The challenge of finding synthetic glucocorticoids with specificity and potency, but without significant adverse events, has led the drive to develop dissociated GR ligands – that is ligands which differentially induce transrepression rather than transactivation. Transrepression is thought to account for the majority of beneficial effects of glucocorticoid therapy (mainly anti-inflammatory effects), while transactivation is associated with adverse effects (e.g. elevated blood glucose levels), however the reality is by no means so clear-cut (Schacke et al., 2002). Multiple pre-clinical compounds are currently under investigation in animal models (De Bosscher, 2010), though the complexity of these physiological and biochemical pathways means that many potential synthetic ligands can no longer be considered as either agonists or antagonists, but rather may have cell or organ specific effects (van der Laan and Meijer, 2008).

1.5 Effects of modulating 5α-reductase on metabolism and the HPA axis.

Against the background above, it is apparent that 5αR is a key enzyme involved in the metabolism of many steroid hormones, including androgens and glucocorticoids. There are several strands of evidence which suggest a

potential link between 5αRs and metabolism, including metabolic effects of enzyme substrates and perhaps products, associations of SNPs in 5αR encoding genes with metabolic disease, and demonstrated alteration of 5αR activity in a range of disease processes. Inhibition of 5αR is a therapeutic approach commonly employed in the treatment of androgen dependent disorders such as benign prostatic hyperplasia. However, reflective of the importance of 5αR in a wide array of processes in health and disease, including glucocorticoid metabolism, there is a potential for 'off-target' effects on metabolic health as a result of 5αR inhibition.

Effects of 5αR inhibitors on extra-prostatic tissues may arise either due to a reduction in 5α-reduced metabolite concentration, an increase in 5αR substrate concentration, or indirect effects as a result of altered clearance and compensatory changes in HPA or hypothalamic-pituitary-gonadal axes. Observations in those with genetic disorders involving 5αR, and in those on drugs inhibiting 5αR are the two investigational modalities in human studies. The former is a very rare scenario, while the latter very common. Genetic defects of 5αR are associated with impaired metabolism of multiple steroid hormones (Fisher et al., 1978, Russell and Wilson, 1994), however metabolic consequences are not described.

1.5.1 Evidence from human studies

The metabolic effects of 5αR inhibitors have been investigated to a limited degree in clinical studies. A meta-analysis investigating the safety of dutasteride (Andriole and Kirby, 2003), showed that there were no significant differences in glucose concentrations or liver function tests after a year of therapy, whether compared with placebo or finasteride. The effect of 1 year of dutasteride treatment on BMI was investigated in a Korean population (Hong et al., 2010). This was a randomised controlled trial with three study arms - tamsulosin alone, dutasteride alone, and combination of tamsulosin and dutasteride. The only measures made were serum testosterone level and BMI. They concluded that, only in those with a lower baseline testosterone level, treatment with dutasteride (whether as monotherapy or in combination) significantly reduced BMI by approximately 0.2 kg/m^2. An underlying mechanism for this finding was not hypothesised.

The effect of finasteride on HDL and lipoprotein lipase levels was investigated in a study of 28 patients with mild BPH (Denti et al., 2000) who were randomised to either placebo or finasteride for 6 months. A significant increase in HDL and lipoprotein lipase levels was seen in the finasteride group, however this did not correlate with DHT concentrations, and the underlying mechanism was not explained. A larger RCT with both

finasteride and dutasteride (Amory et al., 2008) found no difference in lipid profiles after a year of treatment. Overall, while there have been several studies looking at extra-prostatic effects of 5αR inhibitors in humans, detailed assessments of metabolism, and insulin sensitivity in particular, are lacking.

Effects of finasteride on glucocorticoid concentrations and regulation have been explored by some groups. Given the structural similarity of finasteride with endogenous steroids, it has been hypothesised (Rittmaster et al., 1994) that finasteride treatment could alter adrenal steroidogenesis. 10 healthy volunteers were given 28 days of finasteride, with baseline and post-treatment measures. There was no difference in basal or ACTH (250 μg) stimulated cortisol concentrations. Other studies also report no difference in basal cortisol concentrations after 10 days (Lewis et al., 1997) or 6 months (Uygur et al., 1998) of finasteride treatment. A study investigating endocrine effects of finasteride in hirsute women (Fruzzetti et al., 1994) found that basal cortisol decreased significantly after one month of treatment, though this significance was lost after three months. There was also a significant dampening of the response to 250 μg of synthetic ACTH. While these levels and responses were decreased, cortisol values remained within the normal range. The authors hypothesised that perhaps alterations in free versus bound cortisol (and its metabolites) may be taking place, however this was

not investigated further. Effects of dutasteride on the HPA axis are not known, though are the subject of ongoing clinical trials in different areas of research including alcoholism (Covault, 2012) and menstruation related mood disorders (Martinez, 2012).

1.5.2 Evidence from animal models

The role of 5αR1 in rodents can be specifically investigated via genetic manipulation. In comparison to their wild-type counterparts, high-fat fed male mice with targeted disruption of 5αR1 (5αR1 -/-) show significant weight gain, both fasting and post-glucose hyperinsulinaemia and increased liver triglycerides (Livingstone et al., 2008). These changes were not seen in 5αR1 knockout males on a control diet, however were seen in female mice regardless of diet. 11βHSD1 and 5βR, the two other key glucocorticoid metabolising enzymes, did not show an increase in activity, suggesting that the lack of 5αR activity was not compensated for by other enzymes. In contrast to findings of liver fat accumulation and insulin resistance, adipose insulin sensitivity was preserved with appropriate suppression of non-esterified fatty acids with high-dose insulin (Livingstone et al., 2009a).

The administration of finasteride or placebo, with and without gonadectomy, was used to demonstrate both the effects of pharmacological inhibition, as

well as differentiate the relative effects of androgens and glucocorticoids. In obese Zucker rats finasteride (a dual isozyme inhibitor in rats) induced insulin resistance and increased liver triglycerides. Weight and plasma triglycerides remained unchanged, again highlighting the importance of circulating versus intra-tissue metabolic profiles. Changes persisted with or without gonadectomy, supporting the hypothesis of glucocorticoid-mediated changes (Livingstone et al., 2009a).

5αR1-/- male mice have reduced bone mass and decreased forearm muscle grip strength (Windahl et al., 2011), however effects of 5αR1 targeted disruption on muscle insulin sensitivity are unknown.

Basal corticosterone concentrations were unchanged in male 5αR1 knockout mice, however clearance of corticosterone was impaired, after both an acute bolus and chronic infusion of corticosterone in adrenalectomised mice (Livingstone et al., 2010). In the acute setting, a significantly increased level of corticosterone was seen 30 and 60 minutes post-bolus. Chronic infusion was followed by a 2.3 fold increase in plasma corticosterone concentrations. While it could be hypothesised that other steroid metabolising enzymes may compensate for aberrations in 5αR, this was not seen. Activity assays indicated hepatic and gonadal 11βHSD1 and hepatic 5βR were unaltered in 5αR1 knockout mice. In finasteride treated obese Zucker rats, there was no

difference in plasma corticosterone concentrations. Response to suppression or stimulation of corticosterone synthesis was not assessed in response to finasteride treatment.

1.6 Global Hypothesis

Inhibition of 5α-reductase type 1 with the dual isozyme inhibitor dutasteride adversely affects fuel metabolism and causes dysregulation of the hypothalamic-pituitary-adrenal axis.

1.7 Global Aims

To investigate the effects of 5α-reductase inhibitor drugs on metabolism and on the hypothalamic-pituitary-adrenal axis, specifically:

- To establish whether pharmacological inhibition of 5α-reductases has an adverse effect on insulin sensitivity.
- To establish whether metabolic sequelae of 5α-reductase inhibition are more pronounced with inhibition of 5α-reductase type 1 and 2 (with dutasteride), compared to inhibition of 5α-reductase type 2 alone (with finasteride).
- To establish whether inhibition of 5α-reductases causes dysregulation of the hypothalamic-pituitary-adrenal axis.
- In support of these studies, to develop and validate laboratory assays for the measurement of androgens, dutasteride, finasteride and tamsulosin via liquid chromatography tandem mass spectrometry (LC-MS/MS).

Chapter 2: General laboratory methods

2.1 Introduction

Laboratory methods described in this chapter were used in the analysis of samples collected as part of the clinical study described in Chapters 5 and 6. Two novel liquid chromatography tandem mass spectrometry (LC-MS/MS) methods developed and validated are described separately in Chapters 3 and 4. Clinical methods are described in Chapters 5 and 6. Room temperature (RT) is defined as a temperature between 18 - 22 °C.

2.2 Equipment

a) Plate shaker (mass spectrometry and adipokine assays)

IKA® KS 260 Basic shaker (Staufen, Germany)

b) Plate shaker (used in ELISA assays described in Sections 2.3 and 2.4)

Thermo iEMS™ incubator/ shaker (UK)

c) Plate washer (used in ELISA assays described in Sections 2.3 and 2.4)

Anthos Labtec aw1 microplate washer (Salzburg, Austria)

d) Plate reader (used in ELISA assays described in Sections 2.3 and 2.4)

Dynatech Laboratories MRX microplate reader (Berkshire, UK)

e) Centrifuge

Eppendorf 5810R (Hamburg, Germany)

f) Micro-centrifuge

Eppendorf 5415R (Hamburg, Germany)

g) Centrifuge (for 384 well PCR plates)

   Grant-bio LMC-3000 (Grant Instruments Ltd., Shepreth, UK)

h) Nitrogen dry-block (for use with individual glass tubes)

   Techne Dri-Block® DB3A sample concentrator (Staffordshire, UK)

i) Nitrogen dry-block (for use with plates in 96 well configuration)

   SPE Dry™ 96 Dual (Biotage, Uppsala, Sweden)

j) Thermal cycler

   Techne TC-512 (Staffordshire, UK)

k) Real-Time-PCR System

   LightCycler® 480 (Roche Diagnostics Ltd., Burgess Hill, UK), operated with LightCycler® 480 software version 1.5

l) Balance (used for weighing adipose prior to assay in Section 2.10.4, and for weighing charcoal used to strip serum in Chapter 3)

   Mettler HK60 (Mettler Instrumente Ag, Zürich, Switzerland)

m) Balance

   Mettler Toledo MT5 microbalance (Mettler Instrumente Ag, Zürich, Switzerland)

n) Vortex

   Hook and Tucker Instruments rotamixer (Croydon, UK)

o) Sonicator

   Decon® FS-minor sonicator (Ultrasonics Ltd., Sussex, UK)

p) Magnetic stirrer

   SM1 magnetic stirrer (Stuart Scientific Co. Ltd, Surrey, UK)

q) Waterbath (for RIAs)

   Grant Sub14 waterbath (C&M Scientific, Livingston, UK)

r) Homogeniser

   Pro200 homogeniser (PRO Scientific, Oxford, USA)

s) pH meter

   Hanna Instruments pH 210 microprocessor pH meter (Bedfordshire, UK)

t) 1470 Wizard™ Automatic Gamma Counter (Wallac, now Perkin Elmer, Seer Green, UK)

u) 96-Well vacuum manifold

   IST VacMaster®-96 (Biotage, Uppsala, Sweden)

v) 96-Well collecting plates

   96-Well Masterblock® (Greiner Bio One, Frickenhausen, Germany)

2.3 Quantitation of plasma insulin by enzyme-linked immunosorbent assay (ELISA)

Plasma insulin was quantified by a ultrasensitive insulin ELISA kit (DRG®, Mountainside, New Jersey, USA). The manufacturer's limit of detection was 0.07 mU/L. Cross-reactivity with proinsulin and C-peptide was reported as < 0.01%.

Samples assayed were clamp samples (Chapter 5.3.5.6) at timepoints (minutes): +75, +90, +165, +180, +255 and +270. This represented two samples during each steady state phase of the clamp (pre-insulin, low dose hyperinsulinaemia, high dose hyperinsulinaemia). Samples from both clamps for each volunteer were assayed together.

Standards (supplied in the kit) represented a concentration range of 0.15 mU/L – 20 mU/L. Samples were prepared as either neat (baseline, +75, +90); 1:4 in calibrator 0 solution (+165, +180), or 1:8 in calibrator 0 solution (+255, +270). Standards (duplicate; 25 µL) and samples (duplicate; 25 µL) were added to wells already pre-coated with mouse anti-human insulin antibodies. Enzyme conjugate in enzyme conjugate buffer (100 µL; 1:11) was added to each well. After incubation (60 minutes; RT), the plate was washed seven times (300 µL) in wash buffer supplied (diluted 1 in 21 in distilled water) to remove unbound excess conjugate. Tetramethylbenzidine solution was added (200 µL) to each well, the plate was incubated in the dark (30 minutes; RT) before addition of sulphuric acid stop solution (50 µL) to stop the reaction. The absorbance at 450 nm was measured spectrophotometrically. A standard curve was constructed (semi-log power fit with tails standard curve) and sample concentration calculated by interpolation. Acceptable r^2 value of the standard curve was considered to be

> 0.99. Duplicates of standards and samples were deemed acceptable if the relative standard deviation (RSD, calculated as standard deviation/mean*100) was < 20%.

2.4 Quantitation of salivary cortisol by ELISA

Salivary cortisol was quantified by a high sensitivity salivary cortisol ELISA kit (Salimetrics®, State College, Pennsylvania, USA). Standards (supplied in the kit) represented a concentration range of 0.33 - 82.77 nmol/L, with a reported limit of detection of 0.08 nmol/L. Standards (duplicate; 25 μL) and samples (duplicate; 25 μL) were added to wells already pre-coated with monoclonal mouse anti-cortisol antibodies. High (27.3 nmol/L; duplicate; 25 μL) and low (2.9 nmol/L; duplicate; 25 μL) controls were added to separate control wells. Assay diluent (phosphate buffered solution provided with kit) was added (duplicate; 25 μL) to zero and non-specific binding wells. Enzyme conjugate in assay diluent (200 μL; 1:1600) was added to each well. After incubation (60 minutes; RT), the plate was washed four times in wash buffer supplied (diluted 1 in 10 in distilled water) to remove unbound excess conjugate. Tetramethylbenzidine solution was added (200 μL) to each well, the plate was incubated in the dark (30 minutes; RT) before addition of sulphuric acid stop solution (50 μL) to stop the reaction. The difference in absorbance between 630 nm and 450 nm was measured spectrophotometrically. A standard curve was constructed (Sigmoidal semi-

log curve) and sample concentration calculated by interpolation. Acceptable r^2 of standard curve was considered to be > 0.99. Duplicates of standards and samples were deemed acceptable if the RSD was < 20%.

2.5 Quantitation of plasma dehydroepiandrosterone sulphate (DHEAS) by radioimmunoassay (RIA)

DHEAS was quantified using an $^{125}I^-$DHEAS RIA kit (Siemens, Surrey, UK). The manufacturer reported a limit of detection of 0.03 µmol/L, and recommended a standard curve range was 0.14 – 1000 µmol/L. They reported an intra-assay precision of < 6% and inter-assay precision of ≤ 12%. Cross-reactivity with other steroids included: DHEA 0.57%, androstenedione 1.21%, testosterone 0.37%, DHT 0.09%.

Zero calibrator (50 µL; duplicate) was added to the non-specific binding and maximum binding tubes. Remaining calibrators, controls and samples (50 µL; duplicate) were added to DHEAS-Ab coated tubes. $^{125}I^-$DHEAS (1 mL; 1.345 kBq) was added to each well. Following vortex mixing, tubes were incubated in a water bath (37 °C, 30 minutes). Tube contents were decanted, and tubes counted (1 minute) in gamma counter calibrated for ^{125}I. A standard curve was constructed (logit-log curve) and sample concentration calculated by interpolation. Duplicates of standards and samples were deemed acceptable if the RSD was < 20%.

2.6 Quantitation of plasma cortisol by RIA

Plasma cortisol was quantified using a ^{125}I RIA kit (MP Biomedicals, Brussels, Belgium), with samples measured at baseline (fasting sample prior to clamp), following low dose (0.25 mg) dexamethasone suppression (Chapter 5.3.4.5), and at +20, +30, +40 and +60 minutes following IV Synacthen (1 µg, Chapter 5.3.4.5).

Standards (supplied in the kit) represented a concentration range of 0 – 2758.6 nmol/L. The manufacturer reported intra-assay precision was < 9%, inter-assay precision < 10%, and limit of detection 4.7 nmol/L. Volunteers were not receiving synthetic glucocorticoids which may cross-react with the assay, other than prescribed dexamethasone as part of the dexamethasone suppression test; cross-reactivity with dexamethasone was reported to be < 0.1%. Standards, control and samples (duplicate; 25 µL) were added to tubes pre-coated with monoclonal rabbit anti-cortisol antibodies. Cortisol-^{125}I (1 mL; 1.009 kBq) was added to each tube, and vortex mixed prior to incubation (45 minutes, 37 °C). Tube contents were decanted, and tubes counted for 180 seconds in a gamma counter calibrated for ^{125}I. A standard curve was constructed (spline smoothed) and sample concentration calculated by interpolation. Duplicates of standards and samples were deemed acceptable if the RSD was < 20%.

2.7 Quantitation of cortisol binding globulin (CBG) by RIA

Plasma CBG was quantified using a ^{125}I RIA kit (DIAsource ImmunoAssays S.A., Louvain-la-Neuve, Belgium) with samples measured at baseline (fasting sample prior to clamp, Chapter 5.3.4.4).

Standards (supplied in the kit) represented a concentration range of 0 – 0.15 μmol/L. The manufacturer reported intra-assay precision was 3.9%, inter-assay precision 4.8%, and limit of detection 0.005 μmol/L. Samples (singlicate; 100 μL) were diluted in dilution buffer provided (2.4 mL). Standards and controls provided in the kit were reconstituted in distilled water. Standards, controls and diluted samples (singlicate; 100 μL) were added to CBG-antibody (goat anti-mouse) pre-coated tubes. CBG-^{125}I (100 μL; 0.85 kBq) was added to each tube. CBG anti-serum (100 μL) was added to each tube, tubes were gently mixed prior to incubation (2 hours, RT, shaking at 400 rpm). Tube contents were decanted, and tubes were washed (2 mL) with wash solution provided. Wash solution was carefully aspirated and tubes counted for 60 seconds in a gamma counter calibrated for ^{125}I. A standard curve was constructed (sigmoidal) and sample concentration calculated by interpolation.

2.8 Quantitation of plasma Non-Esterified Fatty Acids (NEFAs) by coupled enzyme reaction assay

Plasma NEFAs were quantified by a coupled enzyme reaction assay (Zen-Bio, Inc., Research Triangle Park, USA). Standards, dilution buffer and reagents A and B were provided by the manufacturer. Reagents A and B were reconstituted in manufacturer supplied reagent diluent on the day of the assay. Standards represented a concentration range of 0 – 333 µmol/L. Standards (singlicate; 5 µL, made up to 50 µL with dilution buffer) and serum samples (singlicate; 5 µL) were added to wells. Dilution buffer (supplied in kit; 45 µL) was added to each sample well to result in 1:10 dilution of sample. Reagent A (100 µL) and then reagent B (50 µL) were added to all wells. After gentle shaking the plate was incubated (10 minutes; 37 °C) before being allowed to re-equilibrate (5 minutes; RT). Any bubbles visible in wells were pierced with a needle prior to optical density measurement at 540 nm. A linear standard curve was constructed and sample concentration calculated by interpolation. Acceptable r^2 of standard curve was considered to be > 0.99.

2.9 Measurement of adipokines by multiplex immunoassay

Adipose derived peptides are referred to as adipokines throughout this thesis. Adipokines were measured by Milliplex™, an immunoassay

conducted on the surface of fluorescent-coded magnetic beads. Beads contained two fluorescent dyes; the precise concentration of these dyes was specific to a group of analytes (designated a "panel"), and analyte-specific "capture" antibodies on the surface of the beads conferred analyte specificity and allowed quantitation of multiple analytes simultaneously. The analytes quantified from serum were captured by the appropriate beads, with subsequent addition of a biotinylated detection antibody and a Streptavidin-Phycoerythrin reporter molecule allowing quantitation. Two manufacturer defined "panels" were used, with one assay to measure leptin, MCP-1, and IL-8, and another to measure adiponectin and resistin. Kits and instrumentation were supplied by Merck Millipore (Watford, UK).

2.9.1 Instrumentation

Multiplex assays were performed on a MagPix® system operated with xPONENT® software, version 4.1. The MagPix® system comprised two lasers; the first laser was used to excite internal fluorescent dyes to identify analytes of interest, and the second laser was used to excite the fluorescent reporter molecule. The latter signal was used to quantify the immunoassay on the surface of each bead. The instrument was calibrated prior to each use.

2.9.2 Leptin, MCP-1, IL-8

Samples were analysed according to the protocol in the manufacturer supplied kit. Standards (supplied in the kit) represented a concentration range of 38 – 600000 pg/mL for leptin, 1.3 - 20000 pg/L for MCP-1, and 0.64 - 10000 pg/mL for IL-8. Limits of detection, intra-assay precision, and inter-assay precision reported by the manufacturer 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 on a plate shaker (10 minutes; RT), before removal of assay buffer. Standards or controls (25 µL) were added to appropriate wells, in duplicate. Assay buffer (25 µL) was added to background and sample wells. Serum matrix (lyophilised serum matrix reconstituted in 1 mL deionised water, 25 µL) was added to background, standard and control wells. Samples (25 µL) were added to appropriate sample wells, in duplicate. A suspension of antibody (anti-human) immobilised beads were sonicated (30 seconds) and vortexed (1 minute). 150 µL of each antibody bead vial (one per analyte) was added to mixing bottle, and mixture was diluted up to 3 mL with bead diluent. Prepared magnetic beads (25 µL) were added to each well, the plate was sealed, wrapped in foil and incubated on plate shaker (18 hours; 4 °C). Decanting of well contents in any subsequent steps were performed with assay plate on a magnetic plate. The plate was washed three times with wash

buffer supplied (diluted 1:10 in deionised water, 200 µL/well). Detection antibodies (50 µL) were added to each well. The plate was sealed, covered in foil and incubated on a plate shaker (1 hour, RT). Streptavidin-Phycoerythrin (50 µL) was added to each well. The plate was 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 resuspended 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 of standard curve was considered to be > 0.99.

2.9.3 Adiponectin (total) and Resistin

The method used was identical to that described in Section 2.9.2, with the following exceptions: samples were added at a 1:400 or 1:800 dilution, and no serum matrix was added to background, standard or control wells. Standards (supplied in the kit) represented a concentration range of 0.01 – 160 µg/mL for adiponectin, and 2.56 - 40000 ng/mL for resistin. Limits of detection, intra-assay precision, and inter-assay precision reported by the manufacturer were: adiponectin 145.4 pg/mL, 5.6%, 15%; resistin 6.7 pg/mL, 6%, 13%.

2.10 Quantifying abundance of mRNAs of genes of interest from subcutaneous adipose tissue

2.10.1 Equipment

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

2.10.2 Buffers and Solutions

a) DEPC water

Diethylpyrocarbonate (DEPC; 10 drops) was added to distilled water (1 L). This was mixed, allowed to stand (RT) for 24 hours prior to autoclaving. Storage was at RT.

b) 10x TBE

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

c) 0.5x TBE

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

2.10.3 Tissue collection

A biopsy of subcutaneous abdominal adipose was taken with informed, written consent, as per the clinical study protocol (Chapter 5.3.5.10). Samples were frozen immediately on dry ice and stored at -80 °C until analysis. Biopsies were performed pre-treatment and post-treatment for each volunteer.

2.10.4 RNA extraction from subcutaneous adipose

Adipose tissue (~100 mg) was homogenised in Qiazol Lysis Reagent (1 mL, Qiagen) and incubated (5 minutes, RT). Chloroform (200 µL) was added, mixed and incubated (3 minutes, RT) before centrifugation (12,000 g; 15 minutes; 4 °C). The supernatant (600 µL) was removed, and an equal volume of ethanol (70%, *v/v*) added and mixed by pipetting. The solution was placed in an RNeasy spin column and the eluate discarded after centrifugation (10,000 g; 30 seconds; RT). The column was washed with Buffer RW1 (700 µL) and Buffer RPE (500 µL) sequentially. In each step the eluate was discarded following centrifugation (10,000 g; 30 seconds; RT). Buffer RPE (500 µL) was added to wash the membrane, followed by a further centrifugation step (10,000 g; 2 minutes; RT). The RNeasy spin column was then placed in a fresh collecting tube (2 mL) and remaining Buffer RPE removed by centrifugation (16,000 g; 1 minute; RT). The RNeasy spin column was placed in a fresh Eppendorf (1.5 mL). RNase-free water (40 µL) was

added, incubated (10 minutes, RT) and RNA eluted by centrifugation (10,000 g; 1 minute; RT). The eluate was then added back to the RNeasy spin column, incubated (10 minutes, RT) and RNA re-eluted by centrifugation (10,000 g; 1 minute; RT). Eluted RNA was stored at -80 °C.

2.10.5 RNA quantitation

RNA was quantified using a Nanodrop Spectrophotometer (Thermo Fisher, West Sussex, UK). Concentration was determined by the absorbance at 260 nm wavelength (A260), and the purity assessed by the ratio of RNA/DNA (A260/A280), which was deemed acceptable if between 1.9 and 2.1.

2.10.6 RNA quality

RNA quality was assessed by electrophoresis on a denaturing agarose (Lonza, Berkshire, UK) gel (1.2% *w/v* in 0.5 x TBE). Samples (2 - 3 µL) were prepared by adding loading dye (Promega, WI, USA; 1 in 5 dilution in distilled water; 10 µL). Prepared samples (10 µL) were electrophoresed on the gel (100 V, 1 hr) and RNA integrity assessed on the basis of 28S and 18S ribosomal RNA (rRNA) bands. RNA integrity was deemed satisfactory if clear 28S and 18S bands were present without smearing, and if the 28S rRNA band had approximately twice the intensity of the 18S rRNA band.

2.10.7 Reverse Transcription

Total RNA (0.5 µg) was reverse transcribed using the QuantiTect Reverse Transcription kit. Where insufficient RNA was extracted to allow reverse transcription of 0.5 µg (3 volunteers) the maximum possible (319.2 ng; 394.8 ng; 418.4 ng) RNA was used, and the same amount was reverse transcribed from the volunteer's other sample. RNA (12 µL) was added to gDNA wipeout buffer (2 µL) and incubated (42 °C, 2 minutes) to eliminate any contaminating genomic DNA. To each sample, Quantiscript RT buffer (4 µL), RT primer mix (1 µL) and Quantiscript Reverse Transcriptase (1 µL) were added. Two negative controls were prepared as above, one without reverse transcriptase to detect presence of contamination by genomic DNA (denoted "-RT control"), and one with water instead of RNA to detect any contamination with RNA of reagents used (denoted "water control"). Samples were incubated (42 °C, 15 minutes; then 95 °C, 3 minutes) in PCR thermal cycler, before being cooled at 4 °C. Resultant cDNA was stored at -20 °C.

2.10.8 Real-Time Polymerase Chain Reaction (RT-PCR)

mRNA was quantified using real-time PCR. Intron-spanning primers (shown in Table 2-1 for genes of interest, and Table 2-2 for housekeeping genes, Invitrogen Ltd., Paisley, UK) were designed to match probes within the Roche Universal Probe Library. Aliquots from all samples being analysed

were pooled and prepared as standard curve by serial dilution in PCR grade water at concentrations of: 1:4, 1:8, 1:16, 1:32, 1:64, 1:128; 1:256; 1:512. A Mastermix (8 µL) was prepared consisting of 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), and was added to each well. Where Applied Biosystems (TaqMan®) assay was used instead (Table 2.3), Mastermix (8 µL) was prepared consisting of Probe Master (5 µL), PCR grade water (2.5 µL), primer-probe mix (0.5 µL). cDNA was diluted (1:20) with PCR grade water. A –RT control and water control sample from each batch of reverse transcription was also analysed on every plate. Diluted cDNA (2 µL) was then added to each well. All samples and standards were assessed in triplicate. One gene was quantified for all samples on one plate. The plate was covered and centrifuged (4214 g, 2 minutes, RT) before being run on the PCR system.

Gene: Name **Gene accession number**	**Primer sequence** Forward, 5′ → 3′; Reverse, 3′ → 5′		**UPL Probe**
ACACA: Acetyl-Coenzyme A carboxylase α isoform 1 NM_198834.1	Forward	GATGTGGATGATGGGCTACA	73
	Reverse	TGAGGCCTTGATCATTACTGG	
PLIN2: Adipocyte differentiation-related protein (Perilipin 2) NM_001122.2	Forward	TCAGCTCCATTCTACTGTTCACC	72
	Reverse	CCTGAATTTTCTGATTGGCACT	
ADIPOQ: Adiponectin NM_004797.2	Forward	GGTGAGAAGGGTGAGAAAGGA	85
	Reverse	TTTCACCGATGTCTCCCTTAG	
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	
DGAT2: Diacylglycerol O-acyltransferase homolog 2 NM_032564.3	Forward	GAGGGGTCTGGGAGATGG	55
	Reverse	TTGAGCCAGGTGACAGAGAA	
ESR1: Estrogen receptor α NM_000125.3	Forward	TTACTGACCAACCTGGCAGA	24
	Reverse	ATCATGGAGGGTCAAATCCA	
ESR2: Estrogen receptor β 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	
GR: Glucocorticoid receptor NM_000176.2, NM_001018074.1, NM_001018075.1, NM_001018076.1, and NM_001018077.1	Forward	TTCAAAAGAGCAGTGGAAGGA	11
	Reverse	TTTCTTCGAATTTTATCGATGATG	
LIPE: Hormone sensitive lipase NM_005357.2	Forward	TGCTCGGAATCACAGACACT	90
	Reverse	CAGGTCCATGTTGTGGATGA	
LEP: Leptin NM_000230.2	Forward	TTGTCACCAGGATCAATGACA	25
	Reverse	GTCCAAACCGGTGACTTTCT	

LPL: Lipoprotein lipase NM_000237.2	Forward	ATGTGGCCCGGTTTATCA	25
	Reverse	CTGTATCCCAAGAGATGGACATT	
HSD11B1: 11β-Hydroxysteroid dehydrogenase type 1 (11β HSD1) NM_005525.2	Forward	CAATGGAAGCATTGTTGTCG	20
	Reverse	GGCAGCAACCATTGGATAAG	
GPAM: Glycerol-3-phosphate acyltransferase 1 (mitochondrial) NM_020918.4	Forward	GGAAAGTTTATCCAGTATGGCATT	59
	Reverse	TGATATCTTCCTGGTCATCGTG	
MCP1: Monocyte chemotactic protein 1 NM_002982.3	Forward	TTCTGTGCCTGCTGCTCAT	83
	Reverse	GGGGCATTGATTGCATCT	
PPARG: Peroxisome proliferator-activated receptor γ (PPARγ) NM_005037.5	Forward	TGACAGGAAAGACAACAGACAAAT	7
	Reverse	GGGTGATGTGTTTGAACTTGATT	
PPARGC1A: PPARγ coactivator 1α NM_013261.3	Forward	TGAGAGGGCCAAGCAAAG	13
	Reverse	ATAAATCACACGGCGCTCTT	
SRD5A1: 5α-Reductase type 1 (5αR1) NM_001047.2	Forward	TTGGAGAAATCATGGAGTGGT	37
	Reverse	ACTCTTCAAATTTCCGGAGGTA	
SRD5A2: 5α-Reductase type 2 (5αR2)† NM_000348.3	Forward	TATATTGCGCCAGCTCAGG	80
	Reverse	AAACATACGTAAACAAGCCACCT	
SRD5A2: 5α-Reductase type 2 (5αR2)† NM_000348.3	Forward	CACCTGGGACGGTACTTCTG	29
	Reverse	GCAGTGAGTACACAAATGTCCTG	
SRD5A2: 5α-Reductase type 2 (5αR2)† NM_000348.3	Forward	CAGCTACAGGATTCCACAAGG	50
	Reverse	TCAATGATCTCACCGAGGAA	
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_004599.2	Forward	ATCTGGATCTCGCCAGAGG	74
	Reverse	CCAGGCAGGTTTGTAGGTTG	
UCP 2: Uncoupling protein 2 NM_003355.2	Forward	TGAAAGCCAACCTCATGACA	29
	Reverse	GATGACAGTGGTGCAGAAGC	

Table 2-1 Primers used for genes of interest analysed by real-time polymerase chain reaction with Roche Universal Probe Library (UPL). † SRD5A2 attempted three times with different primers.

Gene: Name Gene accession number	Primer sequence Forward, 5′ → 3′; Reverse, 3′ → 5′		UPL Probe
PPIA: Peptidylprolyl isomerase A (cyclophilin A) NM_021130.3	Forward	ATGCTGGACCCAACACAAAT	48
	Reverse	TCTTTCACTTTGCCAAACACC	
RPL32: Ribosomal protein L32 NM_000994.3	Forward	GAAGTTCCTGGTCCACAACG	17
	Reverse	ATGGCTTTGCGGTTCTTG	
TBP: TATA-binding protein NM_001172085.1	Forward	GAACATCATGGATCAGAACAACA	87
	Reverse	ATAGGGATTCCGGGAGTCAT	
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase NM_002046.3	Forward	AGCCACATCGCTCAGACAC	60
	Reverse	GCCCAATACGACCAAATCC	

Table 2-2 Primers tested as housekeeping genes in real-time polymerase chain reaction with Roche Universal Probe Library (UPL).

Gene: Name	TaqMan ABI catalogue number
CYP19A1: Aromatase	Hs00903413_m1

Table 2-3 TaqMan® Applied Biosystems (ABI) assay was used for aromatase.

Samples were denatured by heating (95 °C, 5 minutes), then underwent 50 cycles of PCR amplification, comprising denaturation (95 °C, 10 seconds), annealing (60 °C, 30 seconds), and elongation (72 °C, 1 second). Samples were then cooled (40 °C, 30 seconds). Amplification curves were plotted (x axis: cycle number; y axis: fluorescence intensity). Excitation was at 483 nm and detection at 533 nm. Triplicates were acceptable if standard deviation of the crossing point was < 0.4 cycles. Standard curve generated from pooled and diluted samples was fitted with a straight line and acceptable if reaction efficiency was between 1.7 and 2.2.

mRNA abundances were quantified and normalised against the mean of the transcript levels of a combination (*PPIA* + *TBP* + *GAPDH*) of house-keeping genes (those tested are summarised in Table 2-2).

2.11 Quantitation of glucose, glycerol, d2-glucose and d5-glycerol by gas chromatography mass spectrometry (GC-MS)

2.11.1 Reagents and standards

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

All solutions for standard and enrichment curves were prepared in HPLC grade water and stored at 4 °C (for a maximum of 2 weeks) until use. Internal standard solution contained $^{13}C_6$-glucose (5 mg/mL) and butanetriol (0.25 mg/mL). Standard solutions prepared were glucose (5 mg/mL), glycerol (0.025 mg/mL), d2-glucose (0.1 mg/mL) and d5-glycerol (0.025 mg/mL). Derivatising solution of pyridine:acetic anhydride (1:1 *v/v*) and the reconstitution solvent (5% acetic anhydride in heptane) were prepared fresh on day of analysis.

2.11.2 Instrumentation

The gas chromatograph mass spectrometer (GC-MS) consisted of a Finnigan GC8000^{TOP} GC, a AS800 autosampler, a single quadropole Voyager mass spectrometer, operated via Xcalibur software version 1.2 (Finnigan, now Thermo Scientific, Manchester, UK). The analytical column was a HP-Innowax column (30 m x 0.32 mm x 0.25 μm; Agilent Technologies Ltd., Stockport, UK). The sample was injected (1 μL) by splitless injection, with injector temperature set to 260 °C. The column oven temperature was 60 °C at injection, programmed to reach 150 °C at a rate of 30 °C/minute, and then further programmed to reach 260 °C at a rate of 10 °C, with a final hold time of 3 minutes. Interface and source temperatures were 240 °C and 175 °C respectively. Helium gas was used as the mobile phase, at a flow rate of 2.5 mL/min. Mass spectra were recorded using negative ion chemical ionisation with methane (research grade, BOC, Edinburgh, UK) as reagent gas at an electron energy of 70 eV. Selected ion monitoring was used with a total run-time of 18 minutes. Derivatives of glycerol, d5-glycerol and butanetriol were monitored from 0 - 10 minutes, and those of glucose, d2-glucose and $^{13}C_6$-glucose monitored from 10 - 18 minutes. Monitored ions were: glycerol triacetate *m/z* 217, d5-glycerol triacetate *m/z* 222, butanetriol triacete *m/z* 231 (internal standard), glucose pentacetate *m/z* 287, d2-glucose pentacetate *m/z* 289, $^{13}C_6$-glucose pentacetate *m/z* 293 (internal standard). Typical retention times for derivatives of glycerol and d5-glycerol were 7.15 and 7.12 minutes

respectively, and butanetriol 8.16 minutes. Glucose, d2-glucose and $^{13}C_6$-glucose derivatives were detected as isomers with typical retention times of 15.06 and 15.38 minutes (glucose derivative) and 15.05 and 15.37 minutes (d2-glucose and IS derivatives).

2.11.3 Sample preparation

A standard curve was prepared in expected concentration ranges of glucose (0 - 9.25 mmol/L), glycerol (0 – 90.5 µmol/L), d2-glucose (0 - 0.18 mmol/L) and d5-glycerol (0 – 90.5 µmol/L) in plasma. 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, Cheshire, UK). Standards, enrichment standards and samples (200 µL) were added. Internal standard solution (25 µL) was added to standard curve and samples. Internal standard was not added to enrichment samples. After a 20 minute incubation (RT), vacuum was applied (0.3 bar) and extracts collected. Eluates were reduced to dryness under oxygen-free nitrogen (OFN; 37 °C). Acetate derivatives were formed (Figure 2.1, Figure 2.2); to the dry residue pyridine: acetic anhydride (200 µL, 1:1, *v/v*) was added and this was allowed to stand (RT, 15 minutes). Derivatised samples were dried under OFN (37 °C) and

reconstituted in 5% acetic anhydride in heptane. 1 µL of sample was injected onto the GC-MS system.

Pyridine:acetic anhydride (1:1, *v/v*)
Room temperature

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

Pyridine:acetic anhydride (1:1, *v/v*)
Room temperature

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

2.12 Quantitation of urinary steroids by gas-chromatography tandem mass spectrometry (GC-MS/MS)

2.12.1 Reagents and standards

Unless stated otherwise, all reagents and standards were from Sigma Aldrich (Dorset, UK). F, E, THF, αTHF, THE, epi-F and epi-THF were from Steraloids (Newport, USA). HPLC grade water was from Fisher Scientific (Loughborough, UK). HPLC grade methanol was from VWR (Lutterworth, Leicestershire, UK). Ethyl acetate was from Rathburn (Walkerburn, UK).

Lipidex™-5000 (PerkinElmer, Waltham, USA) was passed through a sintered glass filter funnel to remove methanol. It was then washed with cyclohexane (200 mL) and mobile phase (98:1:1 cyclohexane:hexamethyldisilazane:pyridine; 200 mL). Solid product was returned to original bottle and prepared into a slurry by addition of mobile phase (approximately 200 mL).

2.12.2 Instrumentation

Samples were analysed by gas chromatography tandem mass spectrometry (GC-MS/MS). The system consisted of a Trace GC Ultra gas chromatograph,

TriPlus Autosampler (room temperature), TSQ Quantum XLS mass spectrometer, operated via Xcalibur software, version 2.1.0 (Thermo Fisher Scientific, Hemel Hempstead, UK).

The analytical column was a DB-17MS column (30 m x 0.25 mm x 0.25 µm; Agilent Technologies Ltd., Stockport, UK). The mobile phase was helium gas at a flow rate of 2 mL/minute. The sample was injected (1 µL) by Programmed Temperature Vaporising (PTV) injection. The column oven temperature was 120 °C initially, and then increased (30 °C/minute) to 200 °C, held for 1 minute, then increased (5 °C/minute) to a final temperature of 300 °C where it was held for 1 minute. The mass spectrometer was operated in positive selected reaction monitoring mode. Auxiliary line and mass spectrometer source temperatures were both 280 °C. Collision gas was argon. Emission current was 50 µA, and electron energy 70 eV. Transitions monitored for quantitation are summarised in Table 2-4. Total run-time was 25.67 minutes.

		Name	Precursor (m/z)	Product (m/z)	CE (V)	Typical R_t (min)
Glucocorticoids	Analytes	Cortisol (F)	605.4	515.4	20	22.98
		Cortisone (E)	531.4	441.4	15	23.73, 23.95
		α-THF	652.6	562.4	15	17.6
		β-THF	652.6	562.4	15	17.83
		THE	578.4	488.3	15	18.67
	ISs	Epi-F	605.4	515.4	20	22.2
		d8-E	539.4	449.4	15	23.62, 23.84
		Epi-THF	652.6	562.4	15	17.42
Androgens	Analytes	Androsterone	360.2	270.2	15	14.71
		Epi-androsterone	360.2	270.2	15	16.14
		Aetiocholanolone	360.2	270.2	15	14.93
	IS	5α-androstane-3α, 17α-diol	331.2	241.2	15	11.51

Table 2-4 Transitions monitored for quantitation. CE, collision energy; V, volts; R_t, retention time; min, minutes; F, cortisol; E, cortisone; TH, tetrahydro-; IS, internal standard. Where 2 isomers were detected (indicated by two R_t), these were combined for quantitation.

2.12.3 Sample preparation

Standards of analytes were prepared in methanol (1 mg/mL). Aliquots (detailed below) were added to water (20 mL) to prepare a series of aqueous standards:

0.05 - 150 μg (epiandrosterone, androsterone and aetiocholanolone; quantified with 5α-androstane-3α, 17α-diol); 0.01 - 300 μg (THF, αTHF, THE; quantified with Epi-THF) and 0.005 - 300 μg (cortisol, cortisone,; quantified with Epi-F and d8-E respectively).

Internal standards (Epi-THF, 30 μg; Epi-F, 5 μg; d8E, 5 μg; 5α-androstane-3α, 17α-diol, 25 μg; total volume 75 μL in acetonitrile) were added to all samples (20 mL) and aqueous standards (20 mL), which were then extracted using conditioned Sep-Pak® C18 cartridges (300 mg, Waters Millipore, Watford, UK). Methanol (5 mL) was added to condition the cartridge and water (5 mL) equilibrate. Blank, standards and samples were added to the cartridges and allowed to elute through with gravity. Cartridges were washed with water (5 mL) and steroids were then eluted with methanol (2 mL). Eluates were reduced to dryness under OFN (60°C).

Steroid conjugates were hydrolysed with β-glucuronidase (Helix pomatia, Type H-2, 85000 units/mL; 100 μL; 37 °C for 48 hours) in sodium acetate buffer (2 mL; 0.2 M; adjusted to pH 4.6 with formic acid). Hydrolysates were

passed through reconditioned Sep-Pak® C18 columns (methanol 5 mL; water 5 mL), eluted with methanol (2 mL). The eluate was dried under OFN (60 °C), reconstituted in water (200 µL) and steroids extracted in ethyl acetate (3 mL).

The supernatant was dried and remaining steroids were derivatised to form methoxime-trimethylsilyl (MO-TMS) derivatives. Fifty µL of methoxyamine hydrochloride in dry pyridine (2% *w/v*) was incubated in capped Reactivial (60 °C; 30 minutes) and then evaporated under OFN (60 °C). 1-(Trimethylsilyl)imidazole (TMSI, 50 µL) was added to the dried residue and incubated (100 °C; 2 hours) in capped Reactivial.

Lipidex 5000 columns were then prepared by the addition of pre-prepared Lipidex 5000 (1 mL in mobile phase) to a Pasteur pipette with silanised glass wool, creating a column depth of ~1 inch. Mobile phase (98:1:1 cyclohexane:hexamethyldisilazane:pyridine) was added (3 x 1 mL).

Mobile phase (1 mL) was added to reaction solution in the Reactivial and then collected and passed through the prepared Lipidex column. The Reactivial was washed with mobile phase (2 x 0.5 mL) and washes were also passed through the Lipidex column. Mobile phase (1 mL) was added directly into Lipidex column. All eluates were collected and evaporated to dryness

under nitrogen (60 °C). Dried samples were reconstituted in 200 µL decane and transferred to GC-MS vials, and stored at -20 °C prior to analysis.

2.13 Quantitation of plasma dexamethasone by liquid chromatography tandem mass spectrometry (LC-MS/MS)

2.13.1 Reagents and standards

HPLC grade water and formic acid were from Fisher Scientific (Loughborough, UK). HPLC grade methanol was from VWR (Lutterworth, Leicestershire, UK). Acetonitrile and ethyl acetate were from Rathburn Chemical Ltd. (Walkerburn, UK). Dexamethasone was from Alfa Aesar (Haysham, UK), 11-dehydrodexamethasone and d4-dexamethasone were from Steraloids (Newport, USA). 6-hydroxy dexamethasone had been synthesised in house (kind gift, Dr G Naredo, Mass Spectrometry Core, University of Edinburgh). Stock solutions of analytes and internal standard were prepared (10 µg/mL in methanol) and stored at -20 °C. Standards were prepared on the day of analysis by serial dilution of stock solutions.

2.13.2 Instrumentation

Analysis was performed on a Waters Acquity™ UPLC with autosampler (10 °C), coupled to an ABSciex QTRAP® 5500 mass spectrometer, and operated with Analyst Software version 1.5.1. Separation was achieved on a SunFire™ C18 column (150 x 4.6 mm, 5 µm) at 20 °C, with a linear gradient from 60:40 to 45:55 (acetonitrile with 0.1% FA: water with 0.1% FA) at a flow rate of 1.5 mL/min with a total run-time of 6 minutes. Ionisation was performed in positive electrospray mode with curtain gas 20 psi, collision gas medium, spray voltage 5500 V, source temperature 500 °C, source gases 40 psi. Mass transitions of protonated ions monitored were (Declustering Potential, DP; Collision Energy, CE; Cell Exit Potential, CXP): dexamethasone *m/z* 393 → 373 (DP 71; CE 11; CXP 16); 11-dehydro dexamethasone *m/z* 391 → 253 (DP 71; CE 27; CXP 12); 6-hydroxy dexamethasone *m/z* 409 → 227 (DP 61; CE 23; CXP 10); d4-dexamethasone *m/z* 397 → 377 (DP 51; CE 11; CXP 16).

2.13.3 Sample preparation

A standard curve was prepared representing a concentration range of 0 – 300 ng/mL for all analytes. Standard curves were prepared in plasma (pooled human plasma collected from men and women ages 16 – 45 years on no medications; TCS Biosciences, Buckingham, UK). d4-Dexamethasone (internal standard, 15 ng) was added to plasma (200 µL) and standard curve, and all samples were extracted with solid-phase and then liquid-liquid

extraction. Initially samples were extracted with Sep-Pak® C18, 200 mg cartridges. Following conditioning (methanol 5 mL) and equilibration (water 5 mL), samples were loaded and then analytes eluted with methanol (2 mL). Eluates were dried under nitrogen (60 °C), resuspended in water (200 µL) and then extracted with ethyl acetate (2 mL). The supernatant was dried and resuspended in mobile phase. Injection volume was 10 µL.

2.14 Routine laboratory tests

Routine laboratory blood tests were sent (on the day of sampling) for processing by the NHS Lothian clinical biochemistry and haematology laboratories at the Western General Hospital (Edinburgh, UK). The laboratory participates in the UK National External Quality Assessment Service to ensure quality control. Sample collection is described in Chapters 5.3.4.3 and 5.3.4.4.

Full blood count was measured by a XE-5000 automated flow cytometer (Sysmex UK, Milton Keyes, UK). HbA1c was measured by reverse-phase HPLC on a Menarini HA8160 HbA1c analyser (Menarini, Wooburn Green, UK). All other analytes were measured on an Architect c16000 analyser (Abbott Diagnostics Ltd., Maidenhead, UK) using manufacturer's kit materials according to laboratory protocols. Low density lipoprotein (LDL)

cholesterol was a calculated parameter, using the equation: LDL cholesterol = total cholesterol – HDL cholesterol – (triglyceride/2.2).

In a single batch at the end of the study, sex hormone binding globulin (SHBG) and oestradiol (E2) were measured by the clinical biochemistry laboratory at the Royal Infirmary of Edinburgh (Edinburgh, UK). SHBG from serum was measured by an automated chemiluminescent based assay according to protocol for, and performed on, an Immulite® 2000 system (Siemens, Surrey, UK). Lower limit of sensitivity was 0.2 nmol/L and intra-assay error ≤ 5.3%. Oestradiol was measured from serum by a Chemiluminescent Microparticle Immunoassay according to protocols from Abbott Diagnostics (Illinois, USA) using an Architect c16000 analyser. The functional sensitivity (minimum concentration quantified with < 20% coefficient of variation) was 50 pmol/ L. Cross-reactivity with 17β-oestradiol 3-sulfate was 0.1% and with oestrone 0.7%, and intra-assay error ≤ 7%.

2.15 Statistics and calculations

All statistical calculations were performed using SPSS for Windows software package, version 19 (IBM, Portsmouth, UK). Statistical significance was taken at the 5% level. Area under the curve calculations were performed with Kinetica, version 5.0 (Thermo Fisher Scientific, Loughborough, UK).

Chapter 3: Measurement of androgens and 5α-reductase inhibitors in human serum by liquid chromatography tandem mass spectrometry

3.1 Introduction

The interpretation of the clinical study described in Chapters 5 and 6 can be enhanced by the measurement of dutasteride, finasteride and androgen concentrations in the serum of participants. Measurement of serum dutasteride and finasteride concentrations can complement assessment of compliance in study volunteers. Measurement of androgen concentrations (testosterone, dihydrotestosterone, androstenedione) is a key component of evaluating pharmacodynamic effects of 5αR inhibitors on androgen metabolism and their possible effects on adrenal and gonadal steroidogenesis. There are no published methods for simultaneous measurement of this combination of analytes from human serum; therefore a novel assay was developed.

Historically androgens such as testosterone have been quantified by radioimmunoassay (RIA), which offers distinct advantages as an analytical method as samples do not require extraction, and methods are fast and cost-effective. However RIAs do also have several important limitations which have seen their use in androgen measurement decline (Soldin and Soldin, 2009). They can be affected by binding proteins e.g. albumin and SHBG (present in unextracted samples) and other structurally related compounds (Tate and Ward, 2004). Accordingly, immunoassays lack precision and accuracy at lower concentrations (Wang et al., 2004a), and this may be

particularly relevant to analytes such as dihydrotestosterone (DHT) which are present in low circulating concentrations (Shiraishi et al., 2008). Furthermore the antibodies for some selected androgens are not specific, giving rise to interference in assays by other hormones (Taieb et al., 2002). Direct comparisons of DHT measurement by immunoassay and liquid chromatography mass spectrometry (LC-MS) show that RIA overestimates DHT concentrations especially at low concentrations, likely a reflection of the lack of analyte specificity with RIA (Shiraishi et al., 2008). For these reasons, the field of Clinical Biochemistry has moved towards the use of LC-MS/MS in assessment of testosterone, particularly in patients with lower concentrations, including women, older men, and patients with type 2 diabetes (Kushnir et al., 2010b). In the study of 5αR inhibitors, the concentration of DHT was anticipated to be suppressed, presenting an analytical challenge.

As banned substances in professional sport, both finasteride and dutasteride are sometimes measured in doping control, as well as in clinical studies, although not routinely in the hospital environment. While the measurement of 5αR inhibitors is usually via chromatography based analytical techniques, two enzyme-linked immunosorbent assays (ELISA) have been developed for the measurement of dutasteride, finasteride and a metabolite of finasteride in human urine (Brun et al., 2010). While potentially promising, at this stage

these assays are still limited in their utility as they have only been validated in urine and are heavily dependent on measurement of 5αR inhibitor metabolites. They have potential issues with cross-reactivity between finasteride and dutasteride (3.7%), although these drugs are unlikely to be co-prescribed. Immunoassay approaches were not suitable for use in the study described, due to the need to separate and quantify multiple steroid and drug compounds in a single assay, both for efficient throughput and limited sample volumes.

The analytical approach selected for this thesis was liquid chromatography tandem mass spectrometry (LC-MS/MS), which largely overcomes many limitations of other analytical methods and allows precise, accurate, and simultaneous determination of multiple analytes present at low concentrations. Gas chromatography mass spectrometry (GC-MS) methods (Legrand et al., 1995, Wudy et al., 1992) may have been possible but requires the additional step of derivatisation which adds to sample handling and preparation time, while also adding potential issues with stability of derivatives. GC-MS methods also tend to have longer run times. The use of high pressure liquid chromatography (HPLC) alone to determine finasteride levels (Ptacek et al., 2000) was not suitable for our purposes as the detection limits offered by HPLC were inadequate to detect analytes present in low concentration. In the absence of mass separation and detection, it may have

been difficult to differentiate compounds which may be co-eluting, for example drug metabolites and isomeric androgens.

An LC-MS approach has previously been used successfully in the quantitation of dutasteride (Gomes et al., 2009, Ramakrishna et al., 2004) and finasteride (Chen et al., 2008, Constanzer et al., 1994, de Menezes et al., 2001, Guo et al., 2007, Matuszewski et al., 1998, Phapale et al., 2010), however these methods have always been for one drug or the other. Measurement of androgens via mass spectrometry has been described by several groups, and of particular interest are those where DHT has been measured via LC-MS/MS and applied to adult male and female (Shiraishi et al., 2008), as well as paediatric populations (Kulle et al., 2010). These assays use small sample volumes (100 µL) with extraction approaches including liquid-liquid (Shiraishi et al., 2008) and solid-phase (Kulle et al., 2010) techniques.

To allow efficient processing of study samples it was desirable to quantify all analytes in a single assay, and no such method has been reported. An extraction method suitable for all analytes was necessary, and especially given the differences in circulating concentrations of the androgenic steroids (e.g. much higher concentration of testosterone than DHT) adequate separation was essential. Expected concentrations of analytes are summarised in Table 3-1. DHT concentrations are decreased with dutasteride

and finasteride therapy by approximately 95% and 70% respectively (Clark et al., 2004, GlaxoSmithKline, 2008, Merck, 1998). In addition, testosterone increases by approximately 10-20% with both 5αR inhibitors, though (unlike DHT) testosterone concentrations generally stay within the normal reference range.

Analyte	Expected circulating concentrations		
	♂ range (ng/mL)	♂ range (nmol/L)	Reference
Androstenedione	0.23 – 1.34 0.37 – 2.41	0.8 – 4.68 1.3 – 8.4	(Kushnir et al., 2010a) (NHSLothian, 2011-2013)
Testosterone	2.65 – 9.71	9.2 – 33.7	(Shiraishi et al., 2008)
DHT	0.14 - 0.77	0.47 – 2.65	(Shiraishi et al., 2008)
DHT post finasteride (↓~70%)	0.04 - 0.23	0.14 - 0.8	(Clark et al., 2004)
DHT post dutasteride (↓~95%)	0.007 - 0.0385	0.02 - 0.13	(Clark et al., 2004)
Finasteride	1.8 - 49; mean trough at steady state 9.4		(Merck, 1998)
Dutasteride	Mean 36 after 3 months treatment		(GlaxoSmithKline, 2008)

Table 3-1 Approximate reference ranges for expected concentrations in serum of androstenedione, testosterone, dihydrotestosterone (DHT) in adult men. Also shown are concentrations of DHT predicted post-treatment with 5αR inhibitors finasteride and dutasteride.

3.2 Aims

To develop, optimise and validate a sensitive, precise and accurate LC-MS/MS method for simultaneous quantitation of testosterone, dihydrotestosterone, androstenedione, finasteride and dutasteride extracted from human serum, with validation based on standards deemed acceptable by the U.S. Food and Drug Administration (USFDA, 2001).

3.3 Method development

3.3.1 Reagents and Standards

Unless stated otherwise, all solvents were from Rathburn Chemical Ltd. (Walkerburn, UK). Water and ammonium hydroxide (35%, *v/v*) solution (both HPLC gradient grade), glacial acetic acid (99.79%, *v/v*) and formic acid were from Fisher Scientific (Loughborough, UK). Methanol and dichloromethane (HPLC gradient grade) were from VWR (Lutterworth, Leicestershire, UK). *o*-Phosphoric acid (85% *v/v*) was from BDH Laboratory Supplies (Poole, UK). Ammonium acetate and ammonium formate were from Sigma-Aldrich (Dorset, UK).

Androstenedione, testosterone, epitestosterone, DHT, dehydroepiandrosterone, 2,3,4-[$^{13}C_3$]-androstenedione, 2,3,4-[$^{13}C_3$]-testosterone and 2,3,4-[$^{13}C_3$]-5α-DHT, were from Sigma-Aldrich (Dorset, UK).

2,2,4,6,6,16,16-[$^2H_7$]-Androstenedione, 2,2,4,6,6-[$^2H_5$]-testosterone, 16,16,17-[$^2H_3$]-testosterone, 2,2,4,4-[$^2H_4$]-5α-DHT and 16,16,17-[$^2H_3$]-5α-DHT were from CDN Isotopes, QMX laboratories (Essex, UK). Finasteride was from Steraloids (Rhode Island, USA) and dutasteride was from AK Scientific (Mountain View, USA). d9-Finasteride was synthesised in-house (kind gift, Dr G Naredo). Stock solutions were prepared at 1 mg/mL and 10 μg/mL in methanol, and stored at -20 °C. Working solutions were prepared on the day of analysis.

3.3.2 Biological samples

For method optimisation and validation, pooled male human serum (collected from healthy men aged 17-45 years on no medications), and steroid-stripped serum were purchased (TCS Biosciences, Buckingham, UK). Due to residual androstenedione and testosterone detectable in the steroid-stripped serum, it was re-stripped before use. Dextran-coated charcoal (Sigma Aldrich, Dorset, UK) was added to steroid-stripped serum (0.1 g/ 10 mL), stirred (~24 hours, 4 °C) and centrifuged (1811 g, 4 °C, 30 minutes). Stripped serum was decanted and then sequentially filtered through 1.20 μm (Sartorius minisart, Sartorius AG, Göttingen, Germany) and 0.22 μm filters (Millex® GP filter unit, Millipore Ireland Ltd., Carrigtwohill, Ireland) until clear serum was obtained. Aliquots were frozen (-20 °C) until further use.

For method application, serum was collected from male subjects participating in the clinical study described in Chapters 5 and 6 prior to and following 90 days of treatment with either dutasteride (*n* = *16*), finasteride (*n* = *16*) or tamsulosin (*n* = *14*). Appropriate ethical approval was obtained prior to sample collection and is described further in Chapter 5.3.3.

3.3.3 LC-MS/MS Instrumentation

Initial method development was performed on a Thermo Electron Surveyor MS Pump and a Thermo Surveyor HPLC autosampler, interfaced with a Thermo Electron TSQ Quantum Discovery triple quadrupole mass spectrometer and operated with Xcalibur Version 2.0.6 software (Thermo Electron, Hemel Hempstead, UK). The method was transferred and developed further on a Waters Acquity™ UPLC (Manchester, UK) with autosampler, coupled to an ABSciex QTRAP® 5500 mass spectrometer (Warrington, UK), operated with Analyst® Software version 1.5.1. Nitrogen was used as source, curtain and collision gas. All subsequent method development and validation steps described were completed on the latter instrument (referred to as 'QTRAP'), which afforded greater sensitivity.

3.3.4 Tuning of standards for ion selection

Solutions (in methanol) of dutasteride (1 µg/mL), finasteride (10 ng/mL), testosterone (100 ng/mL), dihydrotestosterone (100 ng/mL),

androstenedione (200 ng/mL), $^{13}C_3$ testosterone (100 ng/mL), $^{13}C_3$ dihydrotestosterone (200 ng/mL) and $^{13}C_3$ androstenedione (100 ng/mL), d9-finasteride (10 µg/mL) were prepared separately and infused (10 µL/min) into the QTRAP source operating in positive atmospheric pressure chemical ionisation (APCI) mode. Protonated ions were exposed to a range of variables of collision energy, cell exit potential and declustering potential in order to ascertain ideal ionisation conditions. The mass of ions formed was determined and the mass transitions selected were those with the most abundant product ions, from the eight most abundant transitions screened. Mass spectrometric source conditions (curtain gas, collision gas, spray voltage, source temperature, and source gases) were then optimised for DHT, the least abundant analyte. For comparison, the same tuning steps were repeated in electrospray ionisation (ESI) mode.

3.3.5 Chromatographic method development

Using unextracted mixes of analytes in aqueous solution, optimum LC-MS/MS conditions were established. The following parameters were manipulated with variables tested listed:

- Column
    - Kinetex C18 100 x 3 mm, 2.6 µm (Phenomenex®, Macclesfield, UK); Kinetex C18 150 x 3 mm, 2.6 µm (Phenomenex®, Macclesfield, UK); Ascentis express 100 x 3 mm, 2.7 µm

(Supelco, Bellefonte, USA); Kinetex PFP 100 x 3 mm, 2.6 µm (Phenomenex®, Macclesfield, UK); Poroshell C18 100 x 2.1 mm, 2.7 µm (Agilent, Santa Clara, USA)

- Column temperature
    - Ranging from 25 ºC to 50 ºC
- Mobile phase
    - Acetonitrile: water; acetonitrile: water with 0.1% formic acid (FA); methanol: water; methanol: water with FA (0.01%); methanol: water with FA (0.1%); methanol: water with FA (0.2%); methanol: ammonium acetate (AmAc, 5 mM); methanol: AmAc (1mM) with FA (0.1%); methanol: AmAc (2.5 mM) with FA (0.1%); methanol: AmAc (5 mM) with FA (0.1%); methanol: ammonium formate (5 mM).
- Gradient
    - A range of gradients were tried with variations on the initial, peak and interval ratios between the mobile phases. Length of time at each isocratic stage, length of time to peak gradient and length of time returning from gradient were all manipulated.
- Flow rate
    - Ranging from 250 µL/min to 500 µL/min

3.3.6 Optimisation of analyte extraction method from human serum

Types of extraction methods tested included protein crash (Impact™ protein precipitation plate, Phenomenex®, UK), phospholipid crash (Ostro™, Waters, UK), solid phase (Oasis® HLB, 10 mg/ 1 mL, 30 µm, and 30 mg/ 1 mL, 30 µm, both from Waters, UK; Strata-X-Drug B™, 33 µm, cation mixed mode polymer sorbet, 30 mg/ 1 mL, Phenomenex®, UK), supported liquid (Isolute® Array SLE+, 200 µL; Isolute® SLE+ columns, 1 mL, both from Biotage, Uppsala, Sweden) and liquid-liquid extraction. We were unable to replicate the liquid-liquid extraction approach described previously (Shiraishi et al., 2008), with endogenous DHT not detected.

The extraction solvent tested in protein and phospholipid crash methods was acetonitrile, with sample:solvent ratios tested of 1:3, 1:6, and 1:9. In supported liquid extractions, pre-extraction mixing was tested with: formic acid (1%, 0.1%), water, NH_4OH (0.1 M), and acetic acid (0.1%). Extracting solvents tested were: dichloromethane, diethyl ether, ethyl acetate, hexane, methanol, acetonitrile. In liquid-liquid extractions, samples were tested neat or mixed (1:1, *v/v*) with water, or formic acid (0.1%), ammonia (5%). Samples were extracted with a sample:solvent ratio of 1:20 and 1:40. Solvents tested were ethyl acetate:hexane (3:2, *v/v,* with and without NaOH (0.1 M)) and ethyl acetate (with and without saturated NaCl).

The method selected and optimised further was the Oasis® HLB (30 mg/ 30 μm) solid phase extraction method. In an effort to aid deproteination and improve sample flow through the extraction column, mixing of sample was tried with the following: formic acid (2%, 1%, 0.1%), phosphoric acid (4%), water, NH_4OH (0.1 M). The proportion of methanol (in water) in the wash step was optimised, with the following tried: 5%, 15%, 25%, 50%. A second wash/ elution step was also tested consisting of methanol:water (50:50, *v/v*) with either formic acid (2%) or NH_4OH (5%). Elution was tested with methanol, acetonitrile, isopropanol and ethyl acetate.

Matrices tested for standard curve were water, stripped serum, male serum and female serum.

3.3.7 Assay validation

3.3.7.1 Recovery

Recovery was calculated by expressing the mean of the integrated peak areas from extracted standards, as a percentage of the mean integrated peak area from unextracted standards. This was performed in replicates (*n=6*) using stripped serum as matrix and enriched with: androstenedione (1 ng), testosterone (1 ng), finasteride (1 ng), DHT (10 ng) and dutasteride (10 ng).

3.3.7.2 Assessment of ion suppression by serum

The effect of the biological matrix (human serum) on ionisation efficiency was assessed in replicates of 6 by post-spiking extracted blank serum with all analytes amounts corresponding to the midpoint of the standard curve (standard 5, Table 3-2), and response compared to standards with the same amounts of analyte dissolved in mobile phase. Blank serum sample were also run so endogenous analyte amounts could be subtracted from peak areas detected in post-spiked samples.

3.3.7.3 Analyte specificity

Analyte specificity was ensured to avoid potential interferences by other endogenous components in serum. Extracted blank stripped serum analysed with the described method was checked for interferences at or close to the expected retention times for all analytes and internal standards, and this was repeated with unstripped serum for finasteride and dutasteride.

Additional analyte and internal standard specificity was ensured with measurement of two (quantifier and qualifier) mass transitions for all except DHT, where the qualifier transition was not detected in normal serum. Acceptable quantifier: qualifier ratios in biological samples were those within 20% of the mean ratio seen in standards.

3.3.7.4 Limits of Detection (LOD)

LOD were determined by analysing solutions prepared by serial dilution of analyte and internal standard stock solutions, with the LOD corresponding to a peak area where signal: noise ratio (SNR) was ≈ 3.

3.3.7.5 Lower Limits of Quantitation (LLOQ)

LLOQ following extraction was determined by extracting analyte and internal standard from serum at amounts corresponding to 0.2 x LOD, 0.5 x LOD, LOD and 5/3 x LOD. Amounts lower than the LOD were tested in case of ion enhancement effects. The LLOQ was defined as the amount where variance in replicates of 6 was ≤ 20%.

3.3.7.6 Linearity

Two standard curves were generated, one in water (for quantitation of androgens) and one in serum (for quantitation of dutasteride and finasteride). A standard curve was generated with each analyte and internal standard by adding internal standards (1 ng each) to blank serum or water, and increasing amounts of analytes. Standard curves represented concentration ranges: testosterone (1, 2, 3, 5, 7.5, 10, 12.5, 15 ng/mL), DHT (0.1, 0.2, 0.5, 1, 2, 3, 4, 5 ng/mL), androstenedione (0.1, 0.2, 0.5, 1, 2, 3, 4, 5 ng/mL), finasteride (1, 2, 5, 10, 25, 50, 75, 100 ng/mL) and dutasteride (1, 2, 5, 10, 25, 50, 75, 100 ng/mL), and are summarised in Table 3-2. Peak areas of

each analyte and internal standard were integrated and a calibration curve constructed (peak area ratio of analyte/ internal standard versus concentration of analyte). Regression lines of best fit were constructed and deemed acceptable if the regression coefficient, r, was greater than > 0.99. Weightings compared were none, $1/x$ and $1/x^2$ to improve accuracy and precision at the lowest concentrations and to afford intercepts as close to zero as possible.

Std.	AND ng/mL (nmol/L)	Testosterone ng/mL (nmol/L)	DHT ng/mL (nmol/L)	Finasteride ng/mL	Dutasteride ng/mL
0	0 (0)	0 (0)	0 (0)	0	0
1	0.1 (0.4)	1 (3.5)	0.1 (0.3)	1	1
2	0.2 (0.7)	2 (6.9)	0.2 (0.7)	2	2
3	0.5 (1.8)	3 (10.4)	0.5 (1.7)	5	5
4	1 (3.5)	5 (17.4)	1 (3.4)	10	10
5	2 (7.0)	7.5 (26.0)	2 (6.9)	25	25
6	3 (10.5)	10 (34.7)	3 (10.3)	50	50
7	4 (14.0)	12.5 (43.4)	4 (13.8)	75	75
8	5 (17.5)	15 (52.1)	5 (17.2)	100	100

Table 3-2 Points of standard curves; standard curve for androgens prepared in water, standard curves for finasteride and dutasteride prepared in pooled male serum. Std., standard; AND, androstenedione; DHT, dihydrotestosterone.

3.3.7.7 Precision and accuracy

The intra-assay accuracy and precision were determined in a standard curve with 3 points of the standard curve prepared in replicates of 6 (standard points 2, 5, 8). The inter-assay accuracy and precision were determined from four standard curves prepared on different days. The precision was calculated as the relative standard deviation of the mean (RSD) with RSD (%) = standard deviation/ mean x 100. The % accuracy was calculated as the concentration/ theoretical concentration x 100.

3.3.7.8 Stability

Stability was assessed by reinjection of a single calibration curve and patient sample after 24 hours in the auto-sampler (10 °C), and then again following 28 day storage (-20 °C). Storage conditions giving no greater that 10% change in response were accepted.

3.3.7.9 Injector reproducibility

Injector variability was assessed by injecting 6 times the standards 1, 2, 3, 5, 8 (as per Table 3-2), and a pooled male serum sample enriched with both finasteride (10 ng/mL) and dutasteride (20 ng/mL).

3.4 Results

3.4.1 Mass spectrometric conditions of analytes

All analytes and internal standards (structures shown in Table 3-3) ionised efficiently in both positive electrospray ionisation (ESI) and positive atmospheric pressure chemical ionisation (APCI) conditions. APCI was selected for use due to increased peak areas measured for endogenous DHT from serum. Source conditions were optimised to DHT and found acceptable for all other analytes and internal standards. Both quantifier and qualifier transitions of analyte and internal standard were subsequently monitored, with the exception of DHT for which the qualifier ion was not visible in serum therefore was not monitored.

In the final mass spectrometric method, the QTRAP was optimised to, and operated in, positive APCI mode, with curtain gas 25 psi, collision gas low, spray voltage 5 kV, nebuliser current 3.5 µA, source temperature 500 °C, ion source gas 55 psi. Multiple reaction monitoring of the analyte quantifier and qualifier transitions were performed as summarised in Table 3-4.

Analyte structure	IS structure
Androstenedione (MW 286.4)	$^{13}C_3$-Androstenedione (MW 289.4)
Testosterone (MW 288.4)	$^{13}C_3$-Testosterone (MW 291.4)
Dihydrotestosterone (MW 290.4)	$^{13}C_3$-Dihydrotestosterone (MW 293.4)
Finasteride (MW 372.5)	d9-Finasteride* (MW 381.5)
Dutasteride (MW 528.5)	d9-Finasteride* (MW 381.5)

Table 3-3 Analyte and internal standard (IS) chemical structures. MW, molecular weight. *d9-Finasteride was used as IS for both 5αR inhibitor drugs.

	Mass (amu)	Precursor ion (amu)	Product ion (amu) Quan; Qual	Declustering potential (V)	Collision energy (V) Quan; Qual	Cell exit potential (V) Quan; Qual
ANALYTES						
Androstenedione	286.4	287	97; 109	56	29; 27	18; 12
Testosterone	288.4	289	97; 109	16	27; 31	14; 16
Dihydrotestosterone	290.4	291	255	16	21	28
Finasteride	372.5	373	317; 305	26	27; 41	14; 28
Dutasteride	528.5	529	461; 264	161	45; 55	48; 32
INTERNAL STANDARDS						
$^{13}C_3$-Androstenedione	289.4	290	100; 112	31	27; 39	12; 16
$^{13}C_3$-Testosterone	291.4	292	100; 112	1	27; 35	4; 8
$^{13}C_3$-Dihydrotestosterone	293.4	294	258	61	21	12
d9-Finasteride	381.6	382	318; 314	41	33; 41	34; 36

Table 3-4 Mass spectral conditions for analysis of analytes and internal standards utilising atmospheric pressure chemical ionisation. amu, Atomic mass unit; Quan, quantifier ion; Qual, qualifier ion; V, volts.

3.4.2 Selection of internal standards

Stable isotope labelled internal standards were selected. d9-Finasteride was synthesised in house for use as internal standard for both finasteride and dutasteride. d9-Finasteride had an approximately two-fold increase in response in serum compared to water or stripped serum. This was however able to be used in a serum standard curve, as the drugs were not endogenous.

d5-Testosterone and d4-DHT were unsuitable for use as internal standards due to loss of A-ring deuteriums during sample processing. d3-Testosterone was unsuitable for use due to a very high background present in the testosterone transition. d3-DHT and d7-androstenedione were unsuitable for use due to varying response in different matrices; with decreased or increased response respectively in stripped serum and water compared to serum samples. Therefore ^{13}C rather than deuterium labelled standards were selected for use in final method. Suitability and stability of selected internal standards, $^{13}C_3$-androstenedione, $^{13}C_3$-testosterone, $^{13}C_3$-DHT and d9-finasteride was demonstrated, with no significant interference between analytes and internal standards. Stability of all selected internal standards was demonstrated with samples enriched only with internal standard showing acceptable response over time and a lack of analyte seen at amounts of internal standard used. Fragmentation patterns between analytes and

internal standards were essentially identical and their relationship was demonstrated to be linear (Section 3.4.6.5).

3.4.3 Chromatographic conditions

Given the potential for interference between analytes, and also mass + 2 isomers from analytes, it was necessary to develop chromatographic conditions capable of adequately separating all analytes within a single run.

Findings from the parameters assessed during method development were:

- Column
    - A peak for dutasteride was not seen with the Kinetex PFP column, suggesting retention was either too great or too little. Responses for all analytes were seen with the Ascentis express and Poroshell C18 columns, however the peak areas were significantly smaller than seen with the Kinetex C18 columns. At higher concentrations, the 150 mm Kinetex C18 was similar to its 100 mm counterpart, however was superior at lower concentrations with improved signal to noise ratio.
- Column temperature
    - Optimum temperature was found to be 35 ^{o}C, as assessed by peak symmetry and resolution.

- Mobile phase
  - Methanol gave greater peak area than acetonitrile for all analytes. The use of formic acid as a modifier enhanced peak areas achieved with water. A concentration of 0.1% formic acid resulted in larger peak areas than 0.01% or 0.2%. Ammonium acetate (5 mM) with and without formic acid, and ammonium formate did not give significant improvements over formic acid.
- Gradient
  - A gradient was necessary for optimum resolution. The extension of time to reach peak gradient was a the key factor allowing adequate separation of all peaks. This was initially optimised as a 12 minute gradient in aqueous standards, however was later shortened to 9 minutes to allow separation of androstenedione from an interfering peak in serum. This adaptation meant that while testosterone was adequately resolved from DHEA and epitestosterone, the latter two steroids were not clearly resolved from each other. However this was accepted since these steroids were not quantified; the important factor was to ensure they were separated from analytes of interest. The lengthening of the isocratic period

following the gradient resolved issues with DHT giving split peaks.

- Flow rate
    - Optimum flow rate, as judged by peak shape and areas, was 250 µL/min.

A representative chromatogram is shown in Figure 3.1.

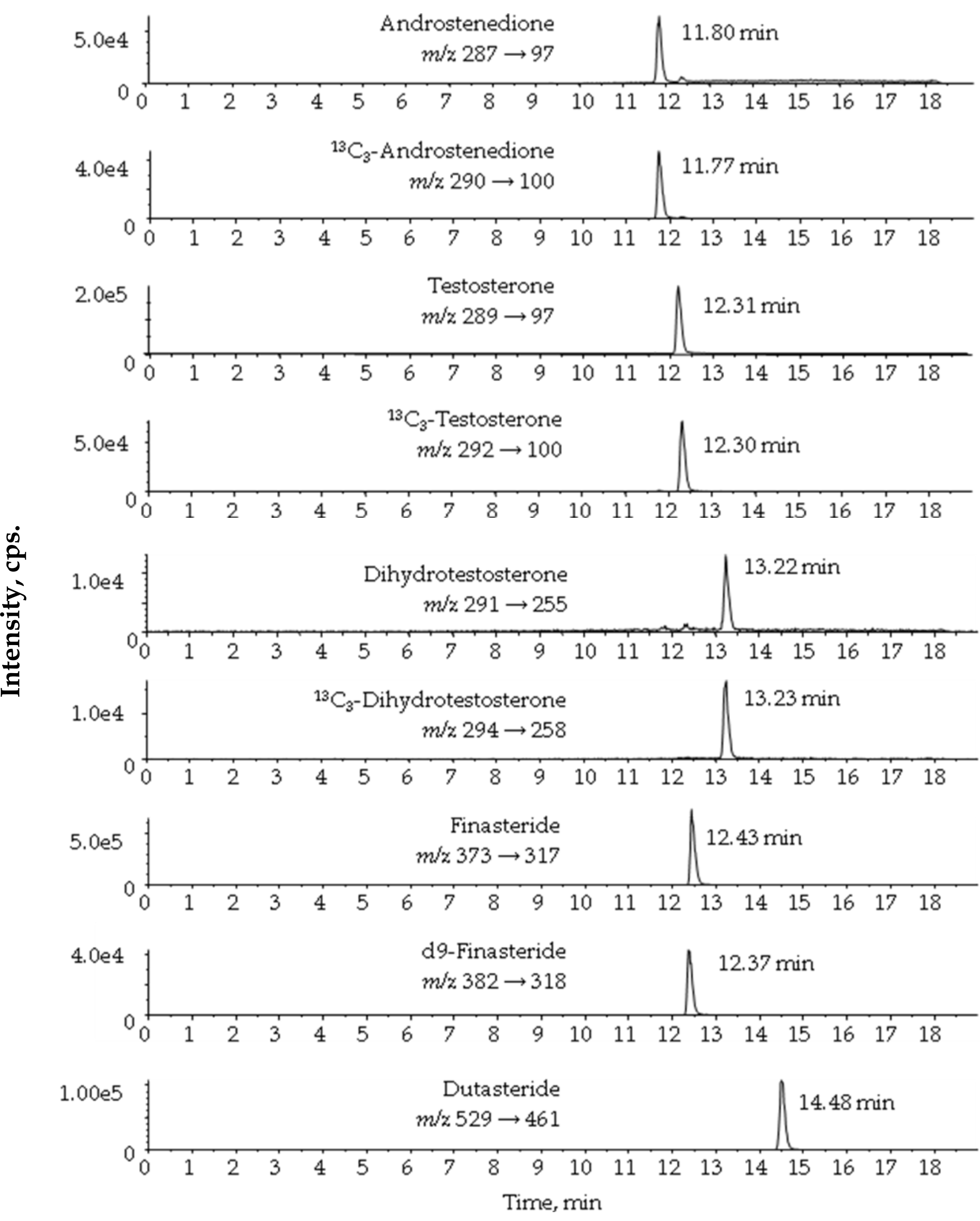

Figure 3.1 Representative chromatogram of quantifier mass transitions for analytes in unextracted standard mix with androstenedione (2 ng/mL), testosterone (7.5 ng/mL), dihydrotestosterone (2 ng/mL), finasteride (25 ng/mL) and dutasteride (25 ng/mL), together with internal standards. Cps, counts per second; min, minutes.

3.4.4 Extraction

While spiked aqueous and stripped serum extracts demonstrated acceptable recoveries from most extraction methods tested, the key challenge in optimising an extraction method was extracting endogenous DHT from serum, and developing one method suitable for extraction of both androgens and 5αR inhibitors.

None of the liquid-liquid extractions tested were able to recover endogenous DHT, and the poor extraction efficiency of the 5αR inhibitors meant they would not have been detected at expected concentrations. Supported liquid-liquid extractions proved the least time-consuming extraction approach, however recoveries and peak areas achieved for 5αR inhibitors were insufficient for expected concentrations, and there were problems with the reproducibility of androgen recovery, therefore this approach was not pursued further. Protein precipitation and phospholipid crash methods gave poorer recoveries and lower peak areas than solid phase extractions, however the main problem was that endogenous DHT was not detected following these extraction methods.

The extraction method which was suitable for all analytes and thus taken forward for further development and validation was a solid phase extraction with Oasis® HLB cartridges. Pre-extraction mixing with water gave the best

response, seen as larger peak areas for DHT. When using only 5% methanol in the wash step there were several large peaks eluting close to the retention time of DHT; optimisation of this step showed 50% methanol in water to retain response of DHT, while acting to wash off the other analytes that were previously detected. Representative chromatograms are shown in Figure 3.2.

Methanol was the best eluting solvent, giving clean extracts and adequate response for all analytes. Isopropanol gave similar responses in all analytes but extracts were visually more opaque. Acetonitrile gave visually more opaque extracts than all other solvents and no improvement in response. Ethyl acetate did improve the peak areas for androstenedione and testosterone by approximately 25%, however did not improve DHT and decreased peak areas for dutasteride by approximately 50%. Given that DHT and dutasteride were the more challenging analytes in this assay, extraction with ethyl acetate was not pursued.

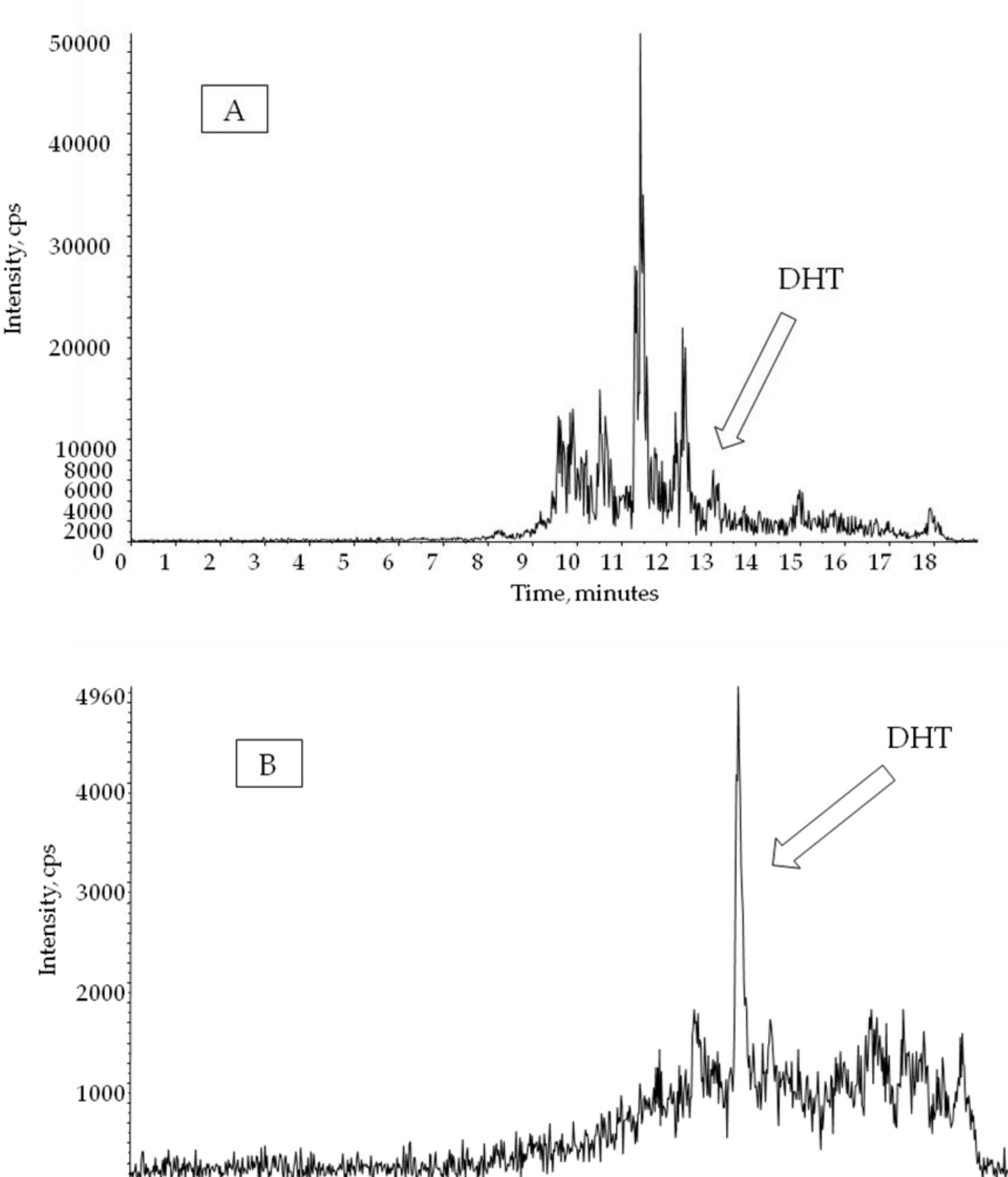

Figure 3.2 Representative chromatograms of wash step optimisation in normal male serum. A. Wash step of 5% methanol in water. B. Wash step of 50% methanol in water, with clearer DHT peak without decrease in size of peak area. DHT, dihydrotestosterone; cps, counts per second.

Significant difficulties were encountered with determining the best matrix for standard curves due to differing responses of both analytes and internal standards in the different matrices tested. During early method development it was noted that analytes responded differently in aqueous versus serum standards in protein crash extraction methods. Following this, stripped serum was used for standard curves. However, several deuterated internal standards differed in their behaviour when extracted from aqueous solution compared to when extracted from serum. Increases in response were seen with d7-androstenedione and d9-finasteride, and a decrease in response was seen with d3-DHT. Female plasma was not suitable for use due to a high concentration of androstenedione. When tested in aqueous extracts, all androgens were able to be accurately quantified in the optimised extraction method. While finasteride was able to be quantified accurately in standards prepared in water, stripped serum or serum, only serum provided accurate dutasteride quantitation. Therefore two standard curves were used – one for androgens and another for the 5αR inhibitor drugs.

3.4.5 Final extraction and chromatographic method taken forward for validation

Samples were extracted via solid-phase extraction (Oasis® HLB). Extraction cartridges were primed with methanol (1 mL) then water (1 mL). Samples (500 µL) were enriched with internal standard (1 ng); water (500 µL) was added. Samples were then mixed with a pipette and loaded onto primed extraction cartridges. After a wash step (50% methanol in water, 1 mL), analytes were eluted with methanol (1 mL). Eluates were dried under oxygen-free nitrogen (37 °C) and reconstituted in mobile phase (100 µL). Injection volume was 10 µL.

Analytes were separated at 35 ºC on Kinetex C18 column (150 x 3 mm, 2.6 µm). Elution was achieved with a 9 minute linear gradient from 30:70 to 80:20 (methanol: water with 0.1% FA) at a flow rate of 250 µL/min. Initial hold was 1 minute with a linear gradient applied until 10 minutes, hold until 16 minutes, with a total run-time of 19 minutes.

3.4.6 Assay validation

3.4.6.1 Recovery

The recoveries (RSD) were: androstenedione 88.7% (15.4%), testosterone 84.6% (13.4%), DHT 85.5% (14.7%), finasteride 89.6% (14.3%), dutasteride 94.5% (10.4%).

3.4.6.2 Ion suppression

There was no ion suppression noted for any analytes, though a small amount of ion enhancement with some. Mean (RSD) relative responses in post-spiked serum compared to unextracted serum were: androstenedione 110.8% (1.7%); testosterone 115.3% (2.1%); DHT 109.2% (2.4%); finasteride 111.0% (0.9%); dutasteride 97.2% (3.0%).

3.4.6.3 Analyte specificity

With the measurement of multiple analytes in a complicated biological matrix such as serum, analyte specificity was an essential developmental aim. All analytes and internal standards were adequately resolved from potential interferences. Of note, testosterone and its biologically inert epi-isomer, epitestosterone, were separated chromatographically, as seen in Figure 3.3. In addition, its isobaric steroid DHEA was not detected in the mass transitions (both quantifier and qualifier) monitored for testosterone. In mass transitions when all three (testosterone, epitestosterone and DHEA) were seen, DHEA co-eluted with epitestosterone, however did not co-elute with testosterone (Figure 3.3).

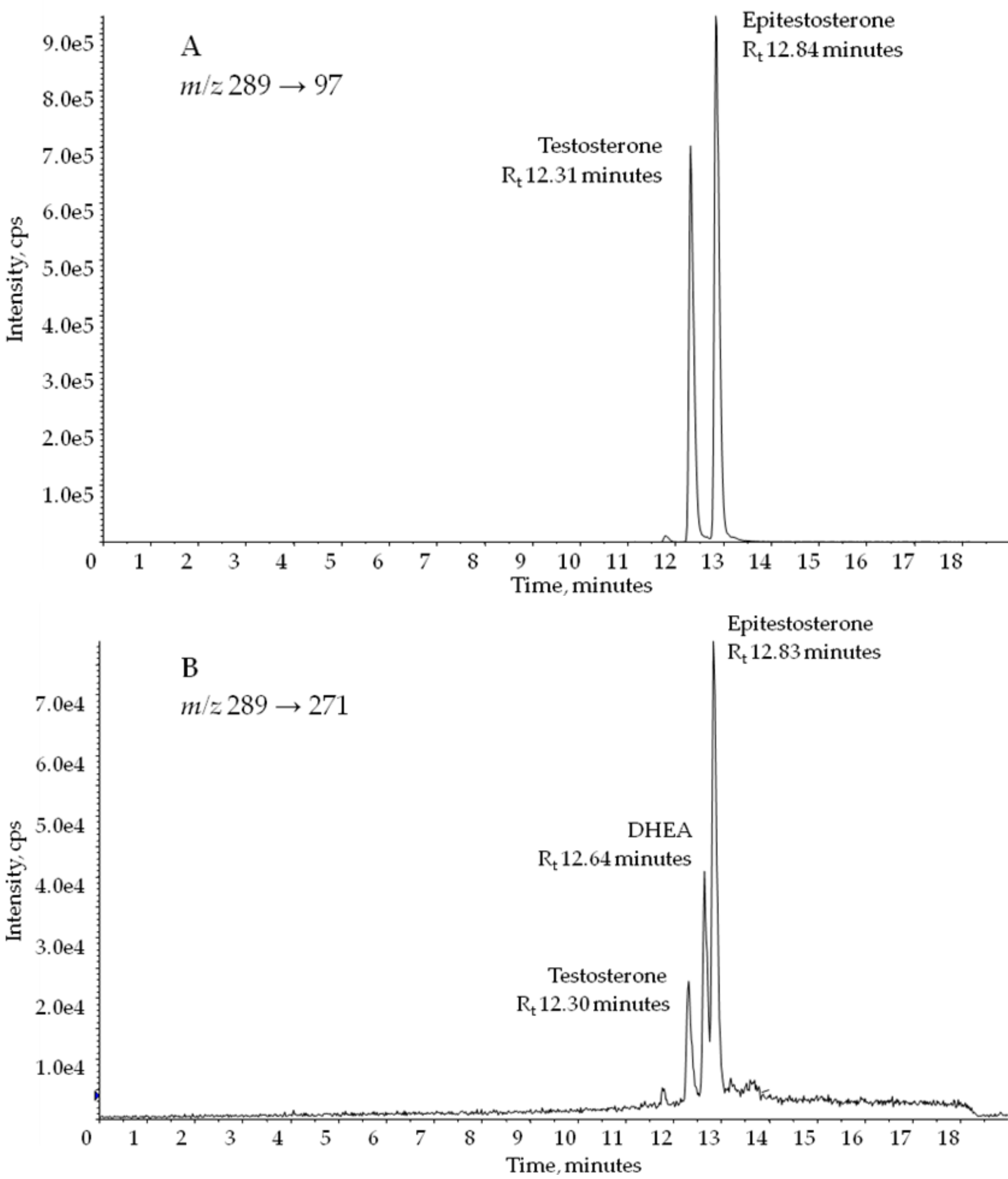

Figure 3.3 Representative chromatograms demonstrating separation of testosterone from epitestosterone and dehydroepiandrosterone (DHEA). A. *m/z* 289→97 had adequate baseline separation of testosterone and epitestosterone; DHEA was not seen in this transition. B. *m/z* 289 → 271 showing adequate baseline separation of testosterone from both DHEA and epitestosterone. While DHEA and epitestosterone were co-eluting, they were separate from testosterone, and DHEA was detected in a different mass transition from testosterone, the analyte of interest. Cps, counts per second; R_t, retention time.

3.4.6.4 LOD and LLOQ

Analyte limits of detection and lower limits of quantitation are summarised in Table 3-5.

	Standard curve range (ng/mL)	LOD (ng/mL)	LOD (nmol/L)	LLOQ (ng/mL)	LLOQ (nmol/L)
Androstenedione	0-5	0.08	0.26	0.125	0.44
Testosterone	0-15	0.003	0.01	0.005	0.02
DHT	0-5	0.13	0.43	0.21	0.72
Finasteride	0-100	0.02		0.003	
Dutasteride	0-100	0.2		0.1	

Table 3-5 Limits of detection (LOD) and lower limits of quantitation (LLOQ) for androstenedione, testosterone, dihydrotestosterone (DHT), finasteride and dutasteride.

3.4.6.5 Linearity

Standard curves were demonstrated to be linear in the range required for all analytes; intercept, r values and weightings are summarised in Table 3-6. Representative standard curves for each analyte are shown in Figure 3.4 (androstenedione), Figure 3.5 (testosterone), Figure 3.6 (DHT), Figure 3.7 (finasteride) and Figure 3.8 (dutasteride).

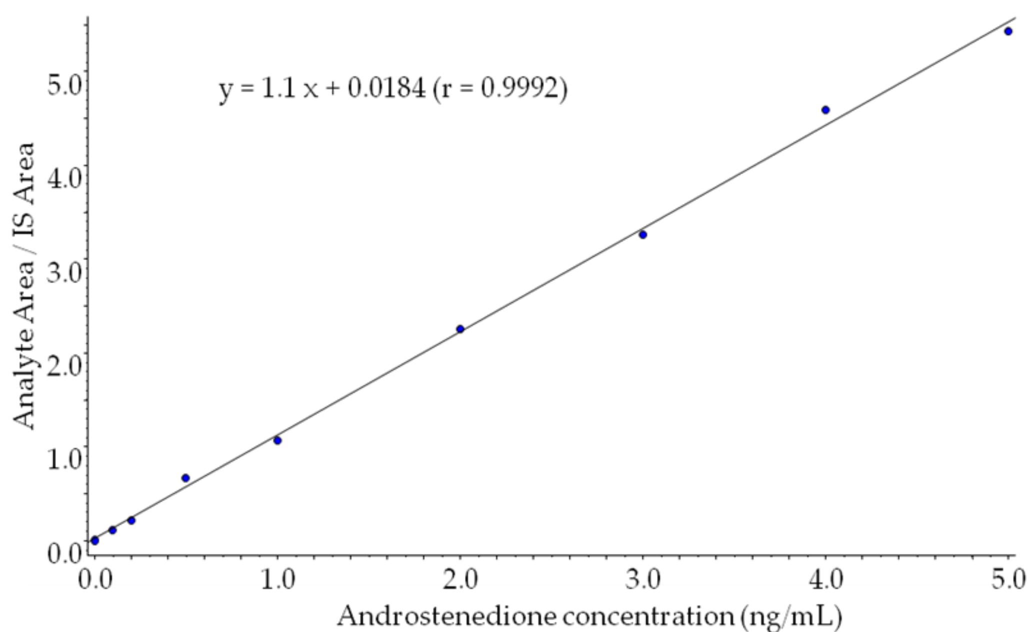

Figure 3.4 Representative standard curve, prepared in aqueous solution, for androstenedione, with no weighting applied. IS, internal standard, $^{13}C_3$-androstenedione.

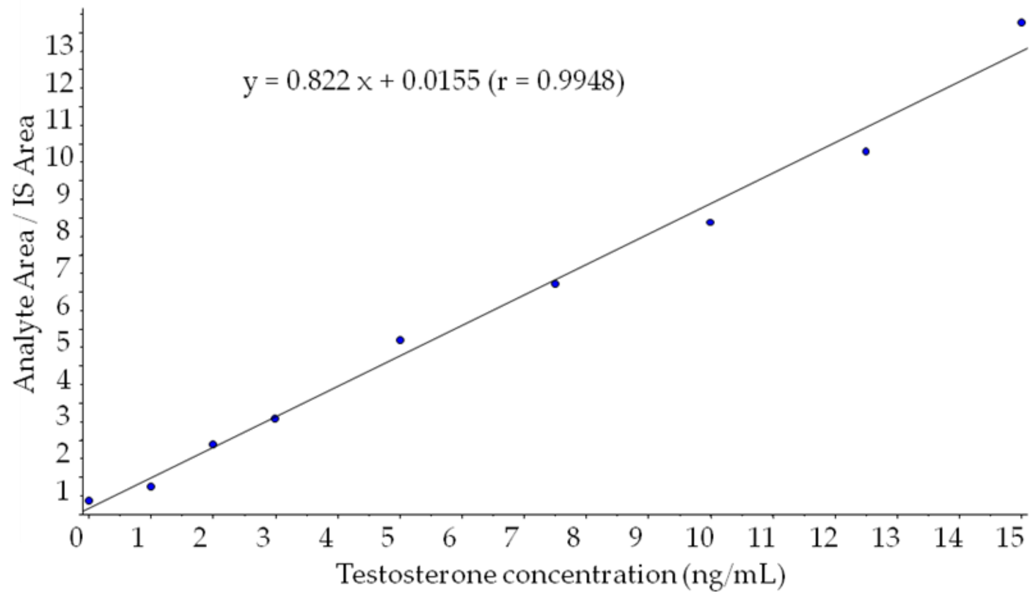

Figure 3.5 Representative standard curve, prepared in aqueous solution, for testosterone, with no weighting applied. IS, internal standard, $^{13}C_3$-testosterone.

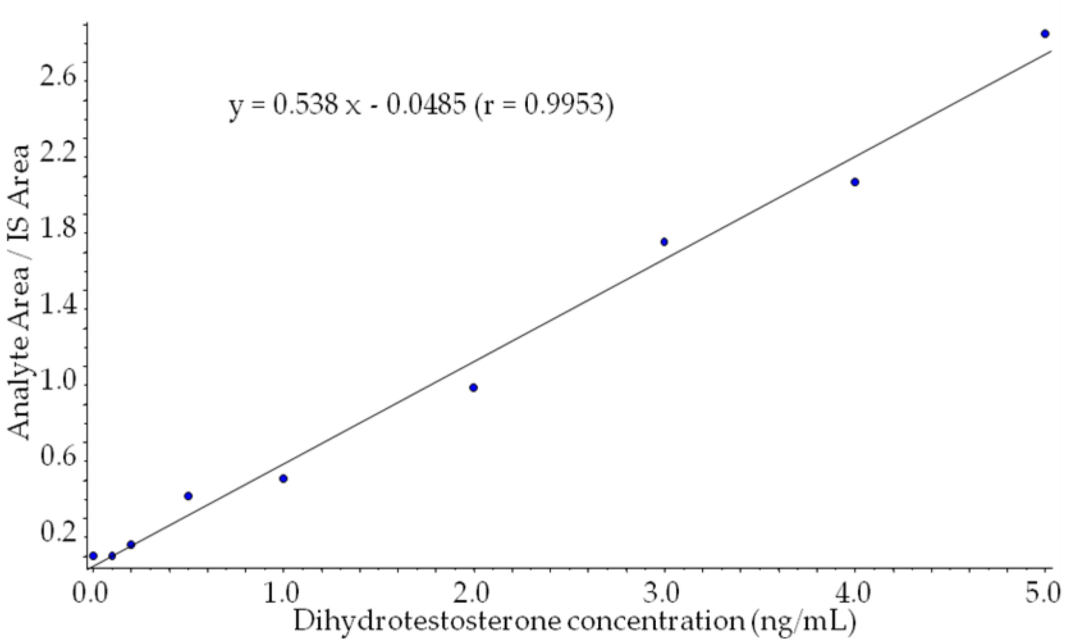

Figure 3.6 Representative standard curve, prepared in aqueous solution, for dihydrotestosterone, with 1/x weighting applied. IS, internal standard, $^{13}C_3$-dihydrotestosterone.

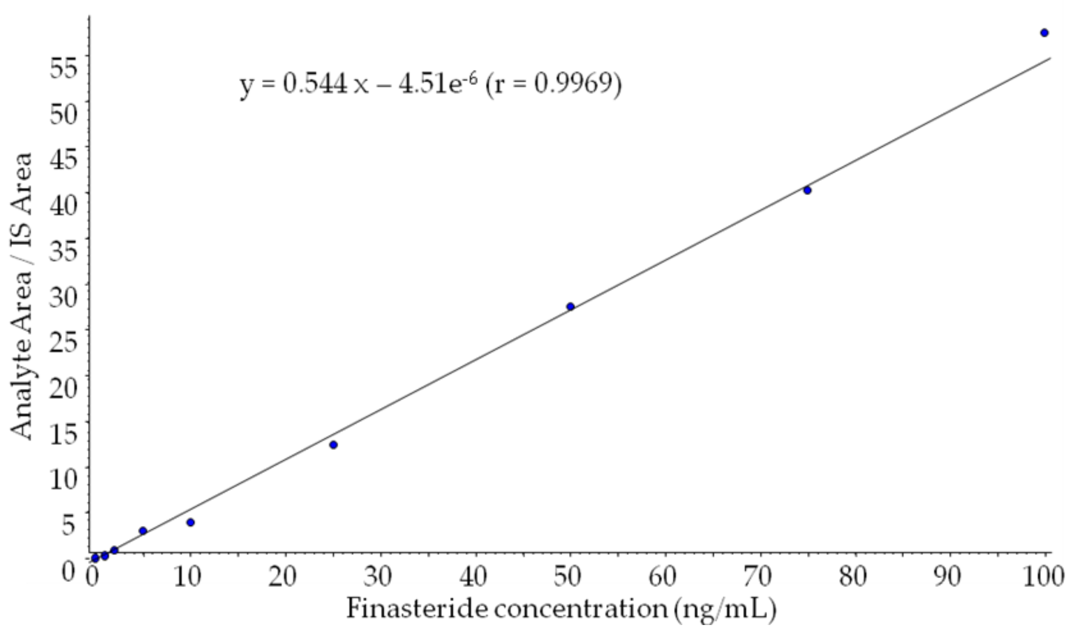

Figure 3.7 Representative standard curve, prepared in serum, for finasteride, with 1/x weighting applied. IS, internal standard, d9-finasteride.

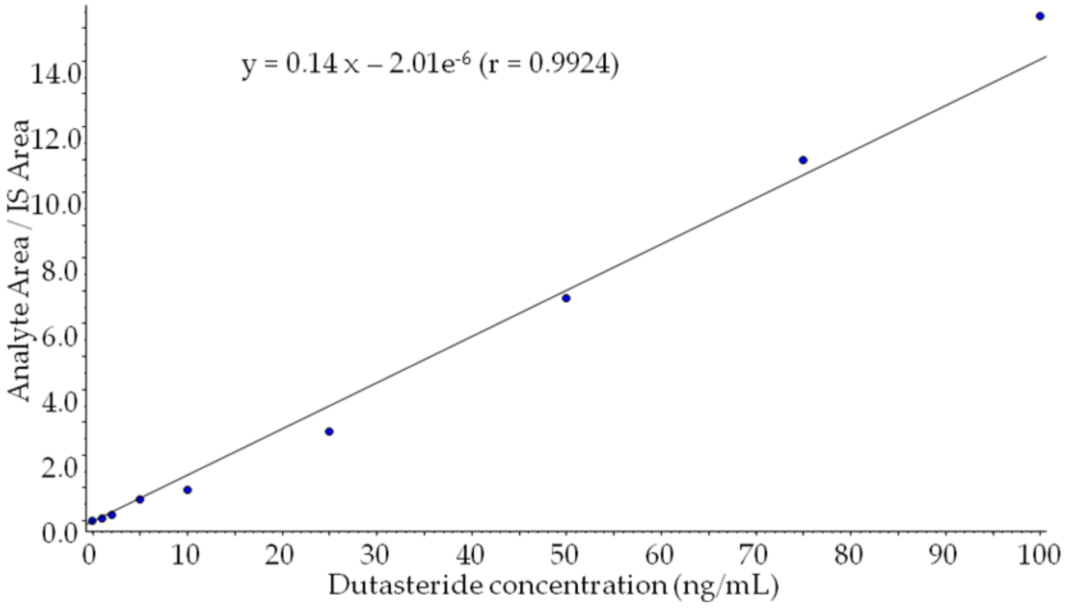

Figure 3.8 Representative standard curve, prepared in serum, for dutasteride, with 1/x weighting applied. IS, internal standard, d9-finasteride.

	Mean intercept (SD)	Mean r value (SD)	Weightings
Androstenedione	-0.00674 (0.02)	0.996 (0.002)	None and 1/x
Testosterone	0.084 (0.31)	0.993 (0.004)	None
Dihydrotestosterone	-0.01097 (0.02)	0.995 (0.003)	1/x
Finasteride	0.044624 (0.06)	0.997 (0.003)	1/x and $1/x^2$
Dutasteride	$8.13E^{-08}$ ($2.42E^{-07}$)	0.993 (0.003)	1/x and $1/x^2$

Table 3-6 Average intercept and r values from standard curves (*n=4*) for each analyte. Weighting used listed in final column. SD, standard deviation.

3.4.6.6 Precision and accuracy

Intra- and inter-assay precision and accuracy are summarised in Table 3-7 and Table 3-8, demonstrating acceptable results for most analytes. The main issues remaining are in the accurate quantitation of low concentrations of DHT, and in variability in accurately quantifying dutasteride in serum.

		Intra-assay (*n=6*)			Inter-assay (*n=3-4*)		
		Concentration (ng/mL): mean (SD)	Precision (% RSD)	Accuracy (%)	Concentration (ng/mL): mean (SD)	Precision (% RSD)	Accuracy (%)
AND	Low (Std 2)	0.18 (0.01)	5.4	87.6	0.19 (0.02)	19.3	93.6
	Mid (Std 5)	1.65 (0.12)	7.2	101	1.75 (0.16)	8.9	92.3
	High (Std 8)	4.22 (0.27)	6.5	98.3	5.01 (0.59)	11.9	103.4
	Sample	0.16 (0.02)	9.9		0.20 (0.04)	20.1	
Testosterone	Low (Std 2)	1.80 (0.07)	2.8	94.4	1.88 (0.19)	10.0	94.9
	Mid (Std 5)	6.50 (0.42)	5.8	117	7.29 (0.83)	11.3	100.6
	High (Std 8)	14.40 (0.75)	4.9	100	15.28 (0.65)	4.2	103.3
	Sample	9.40 (0.61)	6.0		4.22 (0.21)	5.0	
DHT	Low (Std 2)	0.26 (0.02)	9.1	137	0.23 (0.04)	15.5	119.5
	Mid (Std 5)	1.70 (0.11)	6.4	119	1.83 (0.14)	7.4	100.0
	High (Std 8)	4.11 (0.20)	4.9	103	5.00 (0.60)	12.1	105
	Sample	1.39 (0.19)	12.8		1.13 (0.31)	27.3	

Table 3-7 Intra- and inter-assay precision and accuracy data for androstenedione (AND), testosterone and dihydrotestosterone (DHT) quantified from aqueous standard curve.

		Intra-assay (*n*=6)			Inter-assay (*n*=3-4)		
		Concentration (ng/mL): mean (SD)	Precision (% RSD)	Accuracy (%)	Concentration (ng/mL): mean (SD)	Precision (% RSD)	Accuracy (%)
Finasteride	Low (Std 2)	2.18 (0.24)	10	101	1.87 (0.30)	16.2	92.7
	Mid (Std 5)	24.68 (1.34)	5.5	104	24.51 (2.08)	8.5	100.9
	High (Std 8)	95.69 (6.23)	6.5	101	99.17 (4.35)	4.4	102.3
	Sample	8.45 (0.35)	4.1	84.5	8.96 (0.69)	7.8	89.6
Dutasteride	Low (Std 2)	1.67 (0.37)	22	88.2	1.86 (0.16)	8.8	94.5
	Mid (Std 5)	25.07 (2.40)	9.6	110	26.00 (2.60)	10.0	107.4
	High (Std 8)^				86.5 (4.40)	5.1	105
	Sample	16.25 (1.83)	11.2	81.3	15.05 (1.26)	8.4	75.3

Table 3-8 Intra- and inter-assay precision and accuracy data for finasteride and dutasteride quantified from standard curve prepared in serum. ^ Intra-assay data for dutasteride at high standard are incomplete due to a technical failure.

3.4.6.7 Stability

Acceptable autosampler and extract storage stability were demonstrated for a calibration curve and patient sample, as shown in Table 3-9.

	Relative response after 24 hours in autosampler (10 °C)	Relative response after 28 days in freezer (-20 °C)
Androstenedione	102.7%	98.3%
Testosterone	99.7%	101.0%
DHT	90.0%	97.5%
Finasteride	95.3%	103.4%
Dutasteride	92.0%	92.0%

Table 3-9 Relative response for calculated concentrations for all analytes in a single patient sample after 24 hours in the autosampler (10 °C) and after 28 days in the freezer (-20 °C).

3.4.6.8 Injector reproducibility

Reproducibility upon repeat injections (*n=6*) of standards and sample is summarised in Table 3-10 and demonstrated a reasonable contribution of the injector to inter-assay variability. Reproducibility was not improved with repeat injections from vials (instead of plates), therefore for ease of method application, plates continued to be used.

	Std 1	Std 2	Std 3	Std 5	Std 8	Sample
Androstenedione	6.1%	4.5%	7.8%	5.5%	1.8%	6.8%
Testosterone	5.9%	3.6%	1.9%	3.8%	4.2%	2.7%
DHT	116.4%	4.7%	5.2%	8.6%	4.9%	5.7%
Finasteride	4.6%	4.6%	3.4%	5.0%	7.2%	4.5%
Dutasteride	9.7%	5.1%	9.3%	3.1%	6.8%	8.0%

Table 3-10 Injector reproducibility – relative standard deviations (RSD) for each analyte at 5 different extracted standards and a sample spiked with finasteride (10 ng/mL) and dutasteride (20 ng/mL). Of note, dihydrotestosterone (DHT) at standard 1 is below the lower limit of quantitation, and was detected on 4 of the 6 injections only, hence such a high RSD value. Std, standard.

3.5 Application

The assay described was applied to clinical samples, and results are described in Chapter 5.4.3.2.

In addition, a series of 6 male and 6 female serum samples (kindly gifted by Dr Geoff Beckett, Royal Infirmary of Edinburgh (RIE)) which had been analysed by LC-MS/MS were analysed by the described method for comparison. One patient was on testosterone replacement therapy; no patients were on 5αR inhibitor treatment. As androstenedione and DHT are not routinely measured in Dr Beckett's laboratory, these results were unable to be compared. Results are demonstrated in Figure 3.9.

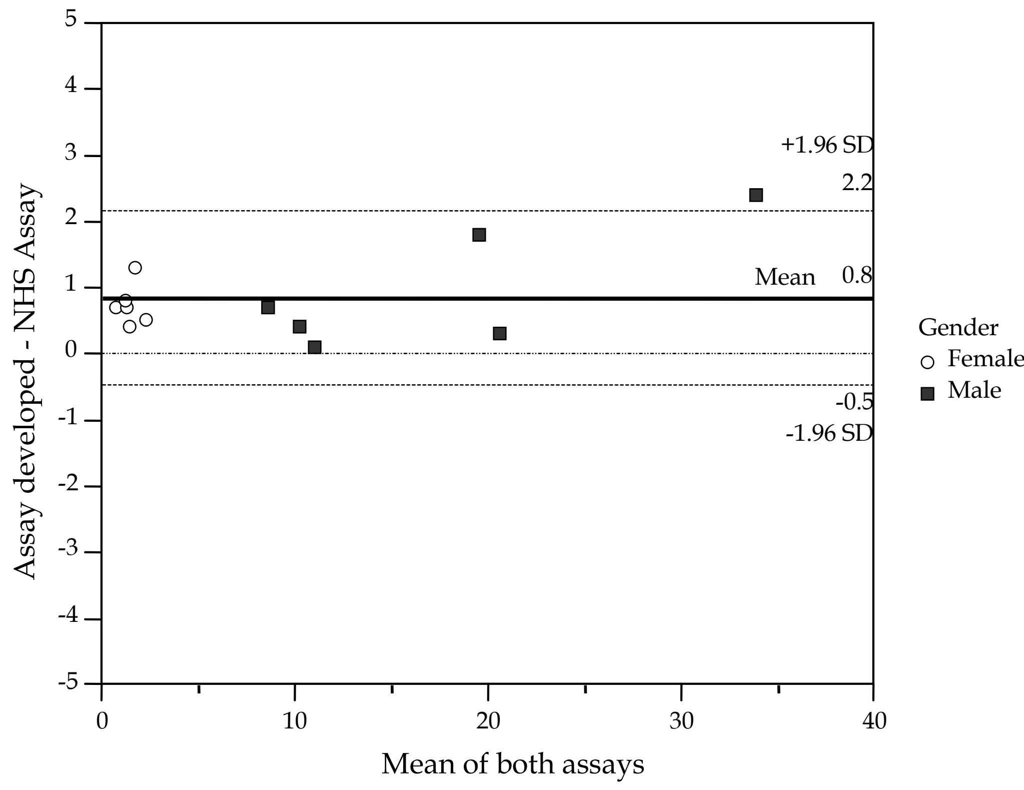

Figure 3.9 Bland-Altman plot demonstrating comparison of serum testosterone concentrations measured by assay presented in this chapter ("Assay developed") with concentrations measured by Dr Beckett's assay ("NHS assay"). Results show that, overall, the assay presented in this chapter gives slightly higher results for serum testosterone.

3.6 Discussion

The purpose of the work presented in this chapter was to develop a method which allowed simultaneous analysis of androgenic steroids and the 5αR inhibitors dutasteride and finasteride from human serum. This approach was applied as a measure of compliance with, and biochemical effects of, 5αR inhibitors (Chapter 5 and 6).

The ionisation methods evaluated for use were electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). All analytes and internal standards ionised in both ionisation modes, and for most ESI was superior in terms of peak area response. However, the main challenge in this assay was the detection of DHT at the low concentrations present in serum. As APCI gave a better response for DHT in terms of greater peak area seen from the same extract, this ionisation mode was selected for further use. For purposes of this assay, this was sufficient for detecting all other analytes, however if the method were to be applied to other applications where DHT was not needing to be measured, then it would be appropriate to use ESI. In addition, if derivatisation was to be used to introduce a permanent positive charge, then ESI would be the appropriate ionisation method for most derivatives. The reason that APCI gave an improved DHT response may be due to its advantage in measurement of small non-polar compounds such as steroids, and particularly due its reduced ionisation of phospholipids and

overall reduced predisposition towards matrix effects (Ismaiel et al., 2008). As seen in the present method also, there was no evidence of matrix ion suppression in APCI.

Method development initially focussed on optimising chromatography, in order to gain adequate separation of peaks and achieve optimal peak areas for each analyte and internal standard. In particular, the potential interference from mass + 2 isomers needed to be avoided through adequate chromatography. Reported methods for detection of individual analytes predominantly use C18 columns, though these vary in length from 50 mm (Chen et al., 2008, Gomes et al., 2009, Ramakrishna et al., 2004) to 150 mm (de Menezes et al., 2001, Guo et al., 2007). Initial attempts were with a pentafluorophenyl (PFP) column, however while adequate peak areas were seen with most analytes of interest, dutasteride was not detected. A possible reason for the poor separation of dutasteride is the 6 fluorine atoms in the dutasteride molecule, which may have a much greater affinity for the column making elution difficult. Rather than improving detection and resolution, the use of a column with a reduced particle size, the Poroshell C18 (particle size 2.1 μm), led to consistently reduced peak areas for all analytes. The best response was with the Kinetex C18 column which allowed all analytes and internal standards to be detected, and resulted in larger peak areas than any other column tested. The 150 mm column provided better peak resolution at

low concentrations than the 100 mm column, and was therefore selected for use.

The mobile phase has a hydrophobic and hydrophilic component, with or without the addition of modifiers. The hydrophobic component in published literature includes acetonitrile (Constanzer et al., 1994, de Menezes et al., 2001, Guo et al., 2007) and methanol (Harwood and Handelsman, 2009, Shiraishi et al., 2008). However the use of acetonitrile suppressed ionisation resulting in smaller peak areas for all analytes. As the hydrophilic mobile phase, water alone can be used (Harwood and Handelsman, 2009), however ionisation was improved when formic acid was added as a modifier. The use of formic acid in conjunction with ammonium acetate has been reported (Chen et al., 2008, Macdonald et al., 2010); this was attempted early in method development with mobile phase switching partway through the run. However, on transfer to the new instrument the mobile phase system went from a quaternary to a binary system which did not allow mobile phase switching in the manner required, though ammonium acetate also no longer significantly improved response from DHT therefore was not pursued further. Gradient was optimised, with extension of the time taken to reach the peak gradient being the key component allowing optimal baseline separation (particularly of testosterone and epitestosterone), while extension

of the isocratic time after the initial gradient resolved issues with peaks splitting.

UPLC was tested briefly, however attempts with both 50 mm and 100 mm C18 UPLC columns showed poorer peak shape and resolution, with no significant increase in sensitivity. This approach was not pursued following initial testing, though it is likely that with further development work this may have yielded superior results and/ or a shortened run-time.

Ideally an internal standard is physically and chemically similar to the analyte of interest, while not being present in the sample being analysed. Deuterium or stable isotope labelled internal standards lend themselves to this application, and being commercially available these are used in many reported androgen assays (Harwood and Handelsman, 2009, Janzen et al., 2008, Shiraishi et al., 2008). While often considered to be the ideal internal standard, deuterated internal standards do have potential problems (Stokvis et al., 2005). During sample processing e.g. in an acidic environment, deuterium may be lost from the steroid structure resulting in unlabelled or partially labelled analyte (Chavez-Eng et al., 2002). With deuterated DHT, instability of deuteriums attached to the steroidal A-ring compared to D-ring has been reported (Boudreau et al., 2009) and in this method authors went on to successfully utilise d3-DHT rather than d4-DHT for DHT quantitation. In

the method presented here this problem was encountered with both d4-DHT and d5-testosterone. Problems with a very high background peak with d3-testosterone (D-ring labelled) meant this was not suitable for use for testosterone quantitation, and instead $^{13}C_3$-testosterone was found to be an appropriate alternative. While d3-DHT did not demonstrate the same instability of deuterium as d4-DHT, it did behave differently in different matrices, making it unsuitable for quantitation, and instead $^{13}C_3$-DHT was successfully employed. Similar problems were found with d7-androstenedione with relative response differing by matrix, therefore this was also replaced with its $^{13}C_3$ labelled counterpart, $^{13}C_3$-androstenedione. Similar fragmentation patterns between analytes and internal standards also provided additional selectivity.

In reported analytical methods for finasteride, a GC-MS technique described by Guarna et al. (Guarna et al., 1995) is notable for the synthesis and use of d3-finasteride as the internal standard. In all other reports of finasteride and dutasteride analysis, non-deuterated internal standards are used, with finasteride used as the internal standard for plasma dutasteride measurement in both published methods (Gomes et al., 2009, Ramakrishna et al., 2004). In-house synthesis of d9-finasteride permitted use of a structurally similar compound as internal standard for both 5αR inhibitors, while the method could be applied to samples without knowledge of a volunteer's

drug assignment. While d9-finasteride gave an exaggerated response in serum, this was not an issue as both dutasteride and finasteride were quantified from a standard curve prepared in serum. The accuracy for dutasteride quantitation was not as high as desired, at 75% (inter-assay) and 81% (intra-assay). This was sufficient for the application in this study, as detection and not accurate quantitation was the principal purpose. It may be that another internal standard (such as a stable-isotope labelled dutasteride) might provide more accurate quantitation, however the assay would need to be developed further to try and improve this.

There are several extraction methods reported for the analytes of interest in the presented assay. The majority of publications report a liquid-liquid extraction (Guo et al., 2007, Harwood and Handelsman, 2009, Janzen et al., 2008, Ptacek et al., 2000, Ramakrishna et al., 2004, Shiraishi et al., 2008), however endogenous DHT was not seen in any of the liquid-liquid extraction methods tested during assay development. Supported liquid extraction initially provided encouraging results for the measurement of androgens, with particularly good response for endogenous DHT. However SLE did not extract 5αR inhibitors with efficiency suitable for clinically expected concentrations. Further problems were encountered when assay validation was attempted with significant intra- and inter-assay variability in response. This was the case for all analytes, however was particularly marked with

androstenedione and DHT, with RSDs in the order of 150%. Attempts were made to troubleshoot this problem, together with input from the manufacturer, however the problem continued therefore SLE could not be pursued further. Success has also been reported with solid-phase extractions for 5αR inhibitors (Constanzer et al., 1994, Gomes et al., 2009) and androgens (Kulle et al., 2010, Mitamura et al., 2005). Waters Oasis® Hydrophilic Lipophilic Balanced (HLB) reversed phase HLB cartridges provided the most reproducible extractions and were effective for all analytes. While the method developed is similar to the extraction method described by Kulle et al. (Kulle et al., 2010), with different instrumentation the same sensitivity was not able to be reproduced. However the extraction method described was simple, and with transfer to 96-well plate format was suitable for processing of large numbers of samples.

A key consideration in this assay was the specificity of the assay. Indeed, this was the principle reason for selection of LC-MS/MS as the analytical method. Androgens were chromatographically well separated, ensuring that isomeric and isobaric species did not affect quantitation. Of note, epitestosterone and DHEA were chromatographically separated from testosterone, and all three androgens being quantified (androstenedione, testosterone and DHT) were chromatographically separated from each other and mass + 2 isomers from analytes or internal standards would not have co-

eluted. In addition, at amounts used, internal standards used did not give any detectable interference in the analyte transitions.

An additional consideration in this assay is the potential presence of 5αR inhibitor drug metabolites in serum. As well as unchanged drug, dutasteride is known to have 3 major (4′-hydroxydutasteride, 1,2-dihydrodutasteride, and 6-hydroxydutasteride) and 2 minor (6,4′-dihydroxydutasteride and 15-hydroxydutasteride) metabolites detected in human serum following dosing to steady state (GlaxoSmithKline, 2008). Finasteride has two main *in vivo* metabolites detected in serum (Merck, 1998). With different molecular weights to their parent drug, metabolites of both finasteride and dutasteride would be anticipated to give rise to different precursor ions and mass transitions and hence not be detected in the current assay.

A crucial consideration is the validity of this assay with respect to expected circulating androgen and drug concentrations (Table 3-1). The main analyte potentially out of range for this assay was DHT, particularly after treatment with 5αR inhibitors, where a dramatic decrease in DHT concentrations is expected. Measurement of DHT via LC-MS/MS is difficult due to its low circulating concentration, poor ionisation and lack of product ions present in high abundance (Kushnir et al., 2011). For these reasons, the majority of approaches in LC-MS have required derivatisation, targeting either the

hydroxyl group at carbon 17 (Licea-Perez et al., 2008, Yamashita et al., 2009), or keto group at carbon 3 (Kalhorn et al., 2007) (Table 3-3). While instrument sensitivity allowed quantitation of normal DHT concentrations in men, in order to quantify DHT post 5αR inhibitor treatment, the method described would need to be developed further and DHT quantified following derivatisation. In addition, for application to other samples where DHT concentrations would be expected to be low and/ or sample volume would be limited (e.g. women, children, small animal models), then derivatisation would also be required. However the method could potentially be applied to studies in female disorders characterised by hyperandrogenism, such as polycystic ovarian syndrome, as circulating testosterone and androstenedione in women would be able to be quantified using this method. For the purposes of application to the clinical study in Chapters 5 and 6, this method was applicable to all samples; however derivatisation would be required if needing to quantify DHT following inhibition of 5αR. The extraction method developed would however still be applicable in this case.

Samples previously assayed for testosterone by Dr G. Beckett (Clinical Biochemstry, RIE) were analysed with the method presented in this chapter. There was overall very good agreement between the assays over a wide concentration range, with a slight positive bias towards towards the assay

presented here. This difference may be related to differences in extraction method; for reasons of economy and speed the RIE laboratory use a protein crash extraction method, which could result in greater ion suppression giving slightly lower calculated concentrations for testosterone.

Measuring 5αR inhibitor concentrations in serum in this study were primarily to act as a supporting assessment of compliance with prescribed study interventions. Detailed pharmacokinetic studies were not the purpose of this assay; however it could be applied to such an application. It was noted throughout assay development that extraction, chromatography and detection of finasteride was achieved with ease in almost every method development test. In contrast, dutasteride was much more difficult to find a suitable extraction and chromatography method for. The assay described is ideal for this setting where measurement of androgens is also desired, and sample volume, handling and analysis time are reduced, however if only 5αR inhibitor drug concentrations are required then an alternate (simpler and shorter) method could be utilised.

The method developed was suitable for measurement of androgens, dutasteride and finasteride from human serum. The assay is notable for the ability to measure these five analytes simultaneously despite significant challenges in chromatographic and extraction method development. The

assay requires relatively little sample volume (500 μL), has a simple extraction method in 96-well format, and is able to quantify DHT without derivatisation. However in order to reliably detect DHT following 5αR inhibition, further method development would be required, including derivatisation. Expected concentrations of all analytes fell within the linear range of the standard curve, other than DHT once its synthesis was inhibited by 5αR inhibitors. Intra- and inter-day precision and accuracy were acceptable, though improvement in accuracy of quantitation of dutasteride and also DHT (at low concentrations) would be desirable. Stability testing demonstrated the assay to be applicable to normal laboratory practice. The described assay was successfully applied to samples taken from research volunteers taking dutasteride, finasteride and control (tamsulosin), as described in Chapters 5 and 6. Attempts to simultaneously measure tamsulosin in this assay were unsuccessful, therefore an additional assay was developed to assess compliance with tamsulosin; this assay is presented in the next chapter.

Chapter 4: Measurement of tamsulosin from human serum by liquid chromatography tandem mass spectrometry

4.1 Introduction

Tamsulosin, an α blocker, is often used as the first-line medical treatment of benign prostatic hyperplasia (BPH) (discussed in Chapter 1.3.2). In clinical studies such as that described in Chapters 5 and 6 where response to pharmacological intervention is studied, establishing compliance with study medication is important. Traditional methods of establishing compliance such as a 'pill count' at the end of the study can be complemented by measurement of presence of drug in serum. The method described in Chapter 3 was not suitable for quantitation of tamsulosin, therefore a separate assay was developed.

Tamsulosin concentrations in those treated with the 0.4 mg modified release formulation are reported to be between 11.8 ng/mL (Ramakrishna et al., 2005a) and 16.1 ng/mL (Taguchi et al., 1998) after a single dose, and 10 ng/mL after 21 days dosing (Wolzt et al., 1998). Measurement of tamsulosin levels has been reported from human plasma by HPLC (Macek et al., 2004, Soeishi et al., 1990), LC-MS (Ding et al., 2002) and LC-MS/MS (Qi et al., 2004, Ramakrishna et al., 2005a, Matsushima et al., 1997, Choi et al., 2012), with key features of these methods described in Table 4-1. HPLC alone precludes additional specificity and sensitivity afforded by its use in conjunction with mass spectrometry, and in all assays described requires undesirably large (1-1.5 mL) sample volumes. While LC-MS methods have been described, it is

now increasingly recognised that tandem mass spectrometry (such as LC-MS/MS) with the monitoring of 2 mass transitions is the gold standard of analyte measurement. As summarised in Table 4-1, the combination of an LC-MS/MS approach, minimal sample volume, simple extraction method and excellent analyte recovery has not been achieved with any published method. Despite relatively low analyte recovery, other features of the method by Ramakrishna et al. (Ramakrishna et al., 2005a) were acceptable with a straightforward and validated method, however this approach was not able to be reproduced on our system, both in terms of chromatography and extraction method, and therefore a new assay had to be developed.

Reference	Analytical method	LLE: pH modifier; solvent	Sample volume	Linear range (ng/mL)	Recovery
(Soeishi et al., 1990)	HPLC	>1 step incl. S.NaHCO3; EA	1.5 mL	0.5 – 15	70%
(Matsushima et al., 1997)	LC-MS/MS	S. NaHCO3; H:EA	200 µL	0.5 – 50	>80%
(Taguchi et al., 1998)	HPLC+	Details NR	NR	0.5 – 50	NR
(Ding et al., 2002)	LC-MS/MS	S. NaHCO3; EA	1 mL	0.2 – 30	84.2-94.5%
(Macek et al., 2004)	HPLC	Na_2CO3; BA	1 mL	0.4 – 40	NR
(Ramakrishna et al., 2005a)	LC-MS/MS	NaOH; DEE:DCM	100 µL	0.1 – 50	59.3%
(Keski-Rahkonen et al., 2007)	LC-MS/MS	S. NaHCO3; EA	1 mL	0.1 – 19.3	66-77%
(Choi et al., 2012)	LC-MS/MS	None; MTBE	500 µL	0.01 - 20	77.8%

Table 4-1 Summary of key characteristics of published assays to quantify tamsulosin from plasma or serum. LLE, liquid-liquid extraction; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry; S., saturated; H, hexane; EA, ethyl acetate; NR, not reported; BA, butyl acetate; DEE, diethyl ether; DCM, dichloromethane; MTBE, methyl *tert*-butyl ether. + This method principally describes a radioreceptor assay which is not included in this table.

4.2 Aim

To develop, optimise and validate a sensitive, precise and accurate LC-MS/MS method for the measurement of tamsulosin from human serum, with validation based on standards deemed acceptable by the U.S. Food and Drug Administration (USFDA, 2001).

4.3 Method development

4.3.1 Reagents and Standards

Unless stated otherwise, all chemicals were from Sigma Aldrich (Dorset, UK) and solvents from Rathburn Chemical Ltd. (Walkerburn, UK). Tamsulosin hydrochloride was from AK Scientific (Mountain View, USA). The internal standard, d9-finasteride had been synthesised in house. Water and ammonium hydroxide (35%, *v/v*) solution (both HPLC gradient grade), and glacial acetic acid (99.79%, *v/v*) were from Fisher Scientific (Loughborough, UK). Methanol and dichloromethane (HPLC gradient grade) were from VWR (Lutterworth, Leicestershire, UK). *o*-Phosphoric acid (85% *v/v*) was from BDH Laboratory Supplies (Poole, UK).

4.3.2 Biological samples

Pooled male human serum (collected from healthy men aged 17-45 years on no medications) was purchased (TCS Biosciences, Buckingham, UK) for use as blank matrix in method development, validation and standard curves. For method application, serum was collected from male subjects (age range 21-73 years, *n=14*) participating in the clinical study described in Chapters 5 and 6. Serum was also analysed from subjects within the study not receiving tamsulosin (age range 20-85 years, *n=33*). All biological samples were collected with informed consent with local regulatory and ethical approval.

4.3.3 Selection of internal standard

In the absence of a commercially available stable isotope labeled internal standard, compounds tested as potential internal standards were trichlormethiazide, ketoconazole, phthalylsulfathiazole (QMX Laboratories, Essex, UK) and d9-finasteride (in-house synthesis, discussed in Chapter 3). These are summarised in Table 4-2.

Internal standards		Name	Structure	MW
N/A		Tamsulosin		408.5
Tested		Trichlormethiazide		380.7
Tested		Ketoconazole		531.4
Tested		Phthalylsulfathiazole		403.4
Tested	Selected	d9-Finasteride		381.6

Table 4-2 Structural formulae and molecular weights (MW) of tamsulosin and compounds tested as internal standard. N/A, not applicable.

4.3.4 LC-MS/MS Instrumentation

Initial method development was performed on a Thermo Electron Surveyor MS Pump and a Thermo Surveyor HPLC autosampler, interfaced with a Thermo Electron TSQ Quantum Discovery triple quadrople mass spectrometer and operated with Xcalibur Version 2.0.6 software (Thermo Electron, Hemel Hempstead, UK). The method was transferred and developed further on a Waters Acquity™ UPLC (Manchester, UK) with autosampler, coupled to an ABSciex QTRAP® 5500 mass spectrometer (Warrington, UK), operated with Analyst® Software version 1.5.1. All subsequent method development and validation steps described were completed on the latter instrument, which offered greater sensitivity.

4.3.5 Tuning of standards for ion selection

Solutions of tamsulosin hydrochloride and d9-finasteride were prepared separately (1 ng/mL in 5:95 methanol: 2 mM ammonium acetate), following dilution of stock solution (1 mg/mL in methanol), and infused (7 µL/min) into the LC-MS/MS source operating in positive electrospray ionisation mode. Protonated ions were exposed to a range of variables of collision energy, cell exit potential and declustering potential in order to ascertain ideal ionisation conditions. The mass of ions formed was determined and the most abundant ions, out of the eight most abundant transitions screened,

undergoing predictable transitions to form abundant product ions were selected for use in subsequent analysis. Both quantifier and qualifier transitions of analyte and internal standard were subsequently monitored. Mass spectrometric source conditions (curtain gas, collision gas, spray voltage, source temperature, source gases) were then optimised for tamsulosin.

4.3.6 Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICRMS)

Accurate mass confirmation for tamsulosin and d9-finasteride were confirmed by FTICRMS. Analyte and internal standard were dissolved separately (20 ng/µL) in acetonitrile: 0.1% trifluoroacetic acid in water (60:40, *v/v*). ESI-FTICRMS was performed by direct infusion into a 12 T SolariX dual source (ESI/MALDI) FTICRMS (Bruker Daltonics, MA, US). The instrument was operated with SolariX control v1.5.0 (build 42.8) software. Ions were detected between *m/z* 250 and 1500, yielding a 1 Mword time-domain transient. Ions of interest were isolated for 20 seconds prior to collision-induced dissociation (CID) experiments. CID was carried out using 35 eV as the collision energy.

4.3.7 Chromatographic method development

Using unextracted mixes of analyte and internal standard in aqueous solution, optimal chromatographic conditions were determined. The following parameters were manipulated, with variables tested listed:

- Column:
    - Variations of stationary phase (C18, PFP), particle size (1.7 µm-5 µm), column dimensions (50-250 mm x 2.1-4.6 mm) and nature (conventional reverse phase, fused core).
    - Columns tested: Ascentis® Express C18 100 x 3 mm, 2.7 µm (Supelco, Sigma-Aldrich, Dorset, UK); Poroshell 120 EC-C18 100 x 2.1 mm, 2.7 µm (Agilent, Wokingham, UK); BDS Hypersil C18, 50 x 2.1 mm, 5 µm (Thermo Scientific, Welwyn Garden City, UK); Hypersil Gold 150 x 2.1 mm, 3 µm (Thermo Scientific, Welwyn Garden City, UK); Atlantis® T3 100 x 2.1 mm, 3 µm (Waters, Elstree, UK); Sunfire™ 250 x 4.6 mm, 5 µm (Waters, Elstree, UK); Sunfire™ 100 x 4.6 mm, 5 µm (Waters, Elstree, UK); Acquity UPLC® BEH C18, 50 x 2.1 mm, 1.7 µm (Waters, Elstree, UK); Kinetex C18 150 x 3mm, 2.6 µm (Phenomenex, Macclesfield, UK); Kinetex PFP 100 x 3mm, 2.6 µm (Phenomenex, Macclesfield, UK).
- Column temperature
    - Ranging from 25 °C to 50 °C

- Mobile phases
    - Methanol: water; Methanol: aqueous ammonium acetate (10 mM, 5 mM, 2 mM, 1 mM, 0.5 mM); Methanol with acetic acid (2%, 1% *v/v*): water; Methanol with acetic acid (2%, 1% *v/v*): aqueous ammonium acetate (5 mM); Methanol: water with formic acid (1%, 0.1%, 0.03% *v/v*); Acetonitrile: water; Acetonitrile: formic acid (1%, 0.1%, 0.03% *v/v*).
- Gradient
    - Isocratic and gradient methods were tested. A range of gradients were tried with variations on the initial, peak and interval ratios between the mobile phases. Length of time at each isocratic stage, length of time to peak gradient and length of time returning from gradient were all manipulated.
- Flow rates
    - Several flow rates were tested ranging from 0.1 – 0.5 mL/min.

4.3.8 Optimisation of extraction method from human serum

Tamsulosin (1 mg) and d9-finasteride (1 mg) were dissolved separately in methanol and stored at -20 °C. Stock solutions (10 µg/mL in methanol) of tamsulosin and d9-finasteride (internal standard) were then prepared and stored at -20 ˚C. Standards of lower concentration were prepared on the day of analysis by serial dilution of stock solutions.

As tamsulosin is not present endogenously, it was appropriate to optimise this assay in the biological matrix, serum. Efficiency of extraction was compared between the different extraction methods attempted, the most effective selected and repetitions (*n*=*6*) performed to ensure reproducibility and robustness.

Types of extraction methods tested included solid phase (Oasis® HLB, 10 mg/1 mL, Waters, UK; Strata-X-Drug B™, 33 µm, cation mixed mode polymer sorbet, 30 mg/ 1 mL, Phenomenex, UK), supported liquid extraction (Isolute® Array SLE+, 200 µL, Biotage, Uppsala, Sweden) and liquid-liquid extraction. In liquid-liquid extractions pre-extraction pH modification was attempted with sodium bicarbonate (saturated, pH 9.55), sodium hydroxide (1 M, pH 13.71; 0.1 M, pH 13.17), ammonium acetate (50 mM, pH 6.65; 10 mM, pH 6.76) and ammonium hydroxide (0.1 M, pH 10.9). Eluting/ extraction solvents of different polarities and lipophilicity were tested with a sample: solvent ratio of 1:20 in all cases, with any additional proportions tested indicated: chloroform, dichloromethane, dichloromethane: diethyl ether (30:70, *v/v,* 1:35), methyl *tert*-butyl ether (MTBE), diethylether, ethyl acetate:hexane (3:2, *v/v*), ethyl acetate, methanol.

4.3.9 Assay validation

4.3.9.1 Recovery

Recovery was calculated by expressing the mean of the integrated peak areas from extracted standards, as a percentage of the mean integrated peak area from unextracted standards. This was performed in 6 replicates with blank serum samples enriched with both analyte (1 ng) and internal standard (1 ng).

4.3.9.2 Assessment of ion suppression

Effect of the biological matrix (human serum) on ionisation efficiency was assessed in replicates of 6 by post-spiking extracted blank serum with tamsulosin (1 ng), and response compared to standards (1 ng) dissolved in mobile phase.

4.3.9.3 Specificity

Analyte specificity was ensured to avoid potential interferences by other endogenous components in serum. Extracted blank serum analysed with the described method was checked for inteferences at or close to the expected retention times for tamsulosin and d9-finasteride. Additional analyte and internal standard specificity was ensured with measurement of two (quantifier and qualifier) mass transitions. Acceptable quantifier: qualifier

ratios in biological samples were those within 20% of the mean ratio seen in standards.

4.3.9.4 Limit of detection (LOD)

Limits of detection (LOD) were determined by analysing solutions prepared by serial dilution of analyte and internal standard stock solutions, with the LOD corresponding to a peak area where signal: noise ratio (SNR) was ≈ 3.

4.3.9.5 Limit of quantitation (LOQ)

Limit of quantitation (LOQ) following extraction was determined by extracting analyte and internal standard from serum at amounts corresponding to LOD, 2 x LOD, and 4 x LOD. The LOQ was defined as the amount where variance in replicates of 6 was ≤20%.

4.3.9.6 Linearity

A standard curve was generated by adding d9-finasteride (1 ng) to blank serum and increasing amounts (0.02, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5 ng) of tamsulosin (corresponding to a concentration range of 0-50 ng/mL). Peak areas of tamsulosin and d9-finasteride were integrated and a calibration curve constructed (peak area ratio of tamsulosin/ d9-finasteride versus amount of tamsulosin). Regression lines of best fit were constructed and deemed acceptable if the regression coefficient, r, was greater than >0.99. Weightings

compared were none, $1/x$ and $1/x^2$ to improve accuracy and precision at the lowest concentrations and to afford intercepts as close to zero as possible.

4.3.9.7 Accuracy and precision

The intra-assay accuracy and precision were determined in a standard curve with 4 points of the standard curve prepared in replicates of 6 (standard concentration: 0.2, 1, 20, 50 ng/mL). The inter-assay accuracy and precision were determined from six standard curves prepared on six independent occasions. Injector variability was assessed by injecting the same samples (0.2, 1, 20, 50 ng/mL). six times on the same day. Precision and injector variability were also assessed in a sample of serum from a patient on tamsulosin treatment. The precision was calculated as the relative standard deviation of the mean (RSD) with RSD (%) = standard deviation/ mean x 100. The % accuracy was calculated as the concentration/ theoretical concentration x 100.

4.3.9.8 Stability

Stability was assessed by reinjection of a single calibration curve and patient sample after 24 hours in the auto-sampler (10 °C), and then again following 28 day storage (-20 °C). Storage conditions giving no greater that 10% change in response were accepted.

4.4 Results

4.4.1 Mass spectrometric conditions of analytes

Tamsulosin and d9-finasteride were both ionised efficiently under positive electrospray ionisation conditions, yielding the mono-protonated molecular ions with *m/z* 409.1 and *m/z* 382.4 respectively. The mass spectrometer was optimised to, and operated in, positive electrospray ionisation mode, with curtain gas 25 psi, collision gas medium, spray voltage of 5 kV, source temperature of 550 °C, source gases both 55 psi. Source conditions optimised to tamsulosin were found acceptable for the internal standard. Selected ionisation was reassessed using the final mobile phase and optimal conditions for analyte and internal standard, and final mass spectrometric conditions are summarised in Table 4-3. Proposed analyte and internal standard fragmentation patterns are shown in Figure 4.1.

	Mass (amu)	Precursor ion (amu)	Product ion (amu)	Collision energy (V)	Cell exit potential (V)	Declustering potential (V)
Tamsulosin (quantifier)	408.5	409	228	33	20	141
Tamsulosin (qualifier)	408.5	409	200	45	16	141
d9-Finasteride (quantifier)	381.6	382	318	31	12	96
d9-Finasteride (qualifier)	381.6	382	314	39	14	96

Table 4-3 Mass spectral conditions for analysis of tamsulosin (analyte) and d9-finasteride (internal standard) utilising positive electrospray ionisation. amu, atomic mass unit; V, volts.

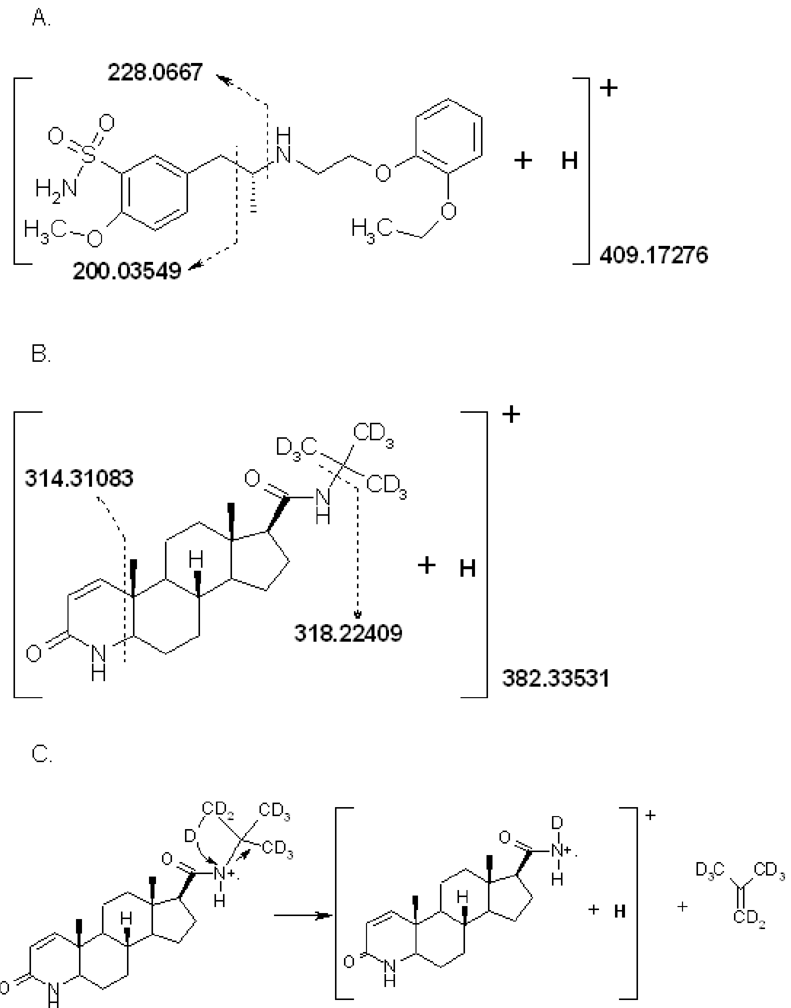

Figure 4.1 Chemical structures and proposed fragmentation patterns for tamuslosin and d9-finasteride. Accurate masses shown for quantifier and qualifier ions were confirmed to within 10 ppm of their theoretical monoisotopic mass by Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. A. Structure and proposed fragmentation pattern for tamsulosin. B. Structure and proposed fragmentation for d9-finasteride. C. Proposed mechanism for fragmentation of d9-finasteride into quantifier ion.

4.4.2 Selection of internal standard

The internal standard selected for use was d9-finasteride due to similar behaviour to tamsulosin in all stages of extraction, chromatography and ionisation. Other internal standards screened were selected from the literature (Ramakrishna et al., 2005b) or by inference of similar structure but were not suitable for use as they ionised very poorly in positive electrospray mode, and required different extraction methods from tamsulosin to ensure reproducible recovery.

4.4.3 Chromatographic conditions

The addition of ammonium acetate to the aqueous mobile phase consistently resulted in increased peak areas for tamsulosin than acidic modifiers, and tamsulosin was not detected with water alone. Mobile phases containing methanol yielded better peak shape than acetonitrile and methanol was therefore selected as non-aqueous mobile phase. Flow rate and column temperature were optimised to enhance peak shape and area for tamsulosin.

4.4.4 Extraction

While a proportion of the analyte added was recovered following extraction by most methods tested (based on methods summarised in Table 4-1), the key developmental difficulties were maximising recoveries and ensuring

reproducibility in recovery. Reproducible recovery could not be obtained by any method tested without pre-extraction pH modification. Upon initial screening, liquid-liquid extraction (LLE) methods were taken forward with pre-extraction pH modification.

4.4.5 Final extraction and chromatographic method taken forward for validation

Serum (100 µL) was dispensed into a glass tube, and d9-finasteride (1 ng) added (as 10 µL of 100 ng/mL solution). NH_4OH (0.1 M, 100 µL) was added and samples mixed (5 minutes, 100 rpm, KS 260 Basic shaker, IKA®, Staufen, Germany). Analyte and IS were extracted via a liquid-liquid extraction with MTBE (2 mL). Following mixing (5 minutes, 100 rpm), and centrifugation (1791g, 4 °C, 10 minutes), the supernatant was transferred to another glass vial. Extracted samples were dried under oxygen free nitrogen (40 °C) and the residue reconstituted in mobile phase (100 µL, methanol: 2 mM ammonium acetate, 5:95, *v/v*). Injection volume was 10 µL.

Compounds were separated at 40 °C on an Ascentis® Express C18 column (100 x 3 mm, 2.7 µm, Sigma Aldrich, Dorset, UK), protected by a Hypersil C18 guard cartridge (10 x 3 mm, 3 µm, Thermo Electron, Hemel Hempstead, UK). Elution with excellent peak shape was achieved with a flow rate of 0.35

mL/min, using a gradient from 5:95 (methanol: aqueous ammonium acetate (2 mM)), with an initial hold of 0.5 minutes then a linear increase in organic mobile phase to 95:5 at 2.5 minutes and held until 3.5 minutes before re-equilibration, with a total run-time of 6 minutes.

Extraction method and chromatographic conditions optimised for tamsulosin were suitable for extraction, elution and detection of d9-finasteride. Representative chromatograms show tamsulosin and d9-finasteride from standard solutions (Figure 4.2) and from a patient sample enriched with only internal standard (Figure 4.3).

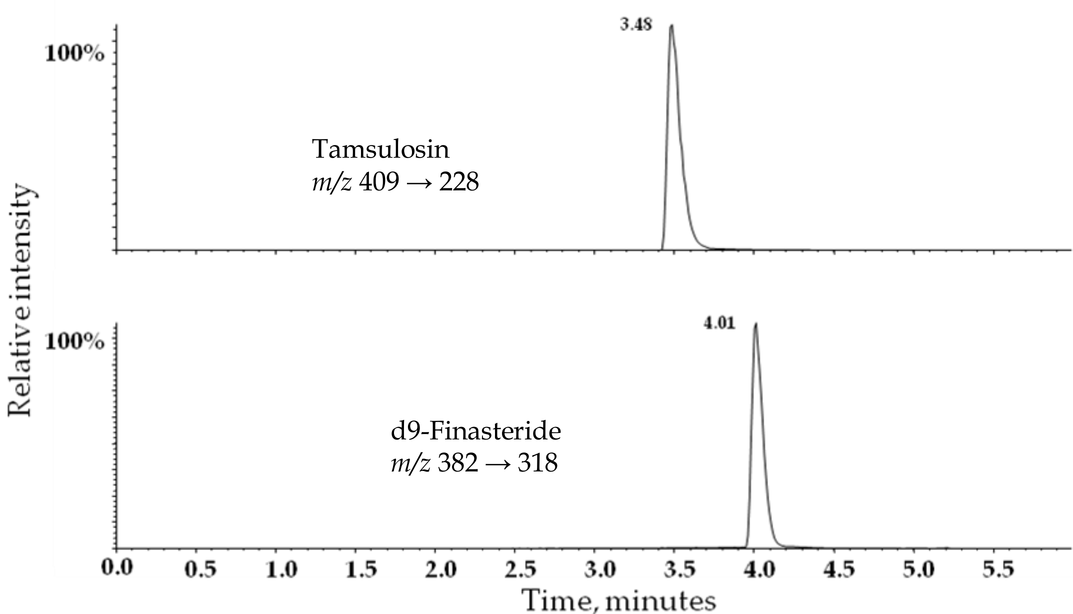

Figure 4.2 Representative mass chromatograms of quantifier mass transitions for analyte, tamsulosin, 20 ng/ mL (upper panel) and internal standard, d9-finasteride, 10 ng/ mL (lower panel) from spiked extracted serum.

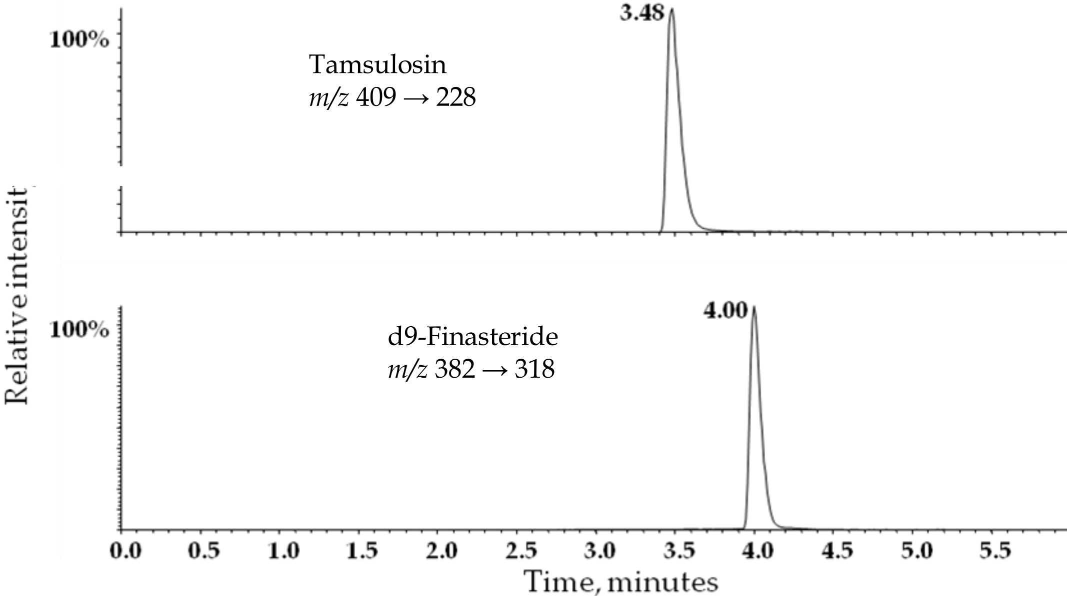

Figure 4.3 Representative mass chromatograms of tamsulosin (quantified as 17.1 ng/ mL) extracted from a patient sample enriched with internal standard, d9-finasteride (10 ng/ mL).The patient had received tamsulosin (0.4 mg daily) for 90 days.

4.4.6 Assay validation

Results from assay validation of LLE are summarised in Table 4-4.

4.4.6.1 Recovery

Analyte recovery was 99.9% (RSD 10.5%) and internal standard recovery was 106.9% (RSD 13.3%).

4.4.6.2 Ion suppression

The presence of matrix did not significantly affect the intensity of response of tamsulosin (94.7% response, RSD 7.8%).

4.4.6.3 Specificity

Extracted blank matrix had no interfering peaks at, or close to, the retention times of tamsulosin or d9-finasteride.

4.4.6.4 Limits of detection (LOD)

Corresponding to a signal: noise ratio of 3, the LOD of tamsulosin was 1.28 pg on column (0.13 ng/mL) and that of d9-finasteride was 1.46 pg (0.15 ng/mL) on column.

4.4.6.5 Limit of quantitation (LOQ)

The intra-assay LOQ following extraction for tamsulosin was 2 pg on column (0.2 ng/mL, RSD 11.1%).

4.4.6.6 Linearity

The standard curve was demonstrated to be linear in the range 0.2-50 ng/mL with an average r value of 0.9952 (*n*=6) with 1/x weighting applied, and average intercept of 0.001 (*n=6)*. A representative standard curve is shown in Figure 4.4.

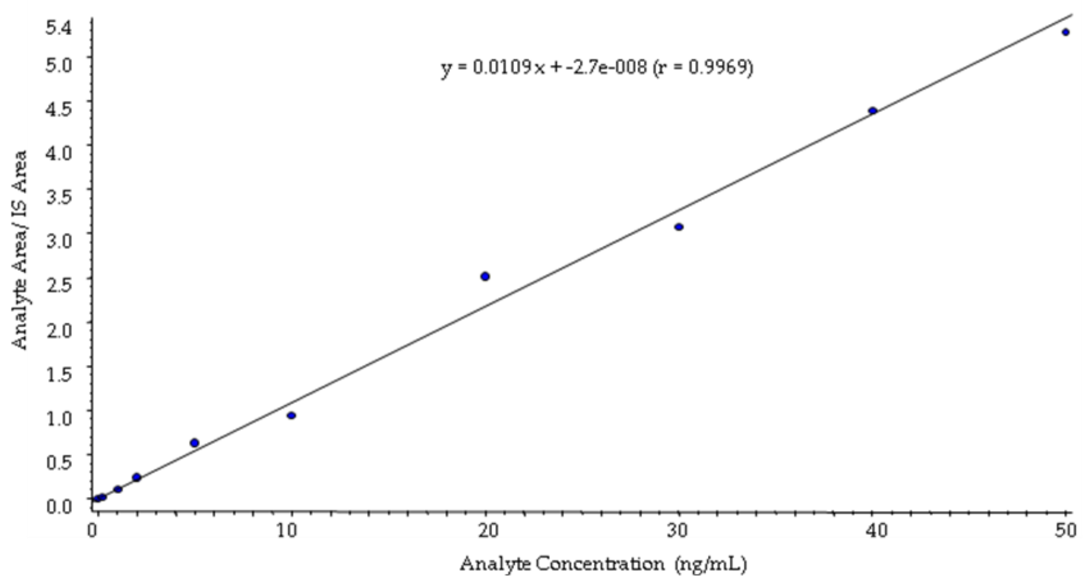

Figure 4.4 Representative standard curve with 1/x weighting applied. IS, internal standard.

4.4.6.7 Precision and Accuracy

Intra and inter-assay accuracy and intra-assay precision were acceptable (<20% RSD for precision and 80-120% accuracy) at the LOQ, and inter-assay

precision was acceptable from the low point (1 ng/mL) of the standard curve (RSD 7.9%). Above these values, variability relating to precision (RSD) and accuracy was <15% and were therefore acceptable. Results are summarised in Table 4-4.

4.4.6.8 Stability

Acceptable autosampler and extract storage stability were demonstrated (Table 4-5). Data collated from a single standard curve and patient sample reinjected following 24 hours in the autosampler (10 °C) were unchanged, with a relative response (stored/original) of 100.3%. Extracts reinjected following storage at -20 °C for 28 days had 93.9% response compared to the original run.

4.4.6.9 Injector reproducibility

Acceptable reproducibility upon repeat ($n=6$) injections of standards and sample was demonstrated with RSDs of: LOQ (0.2 ng/mL) 6%, low (1 ng/mL) 3%, mid (20 ng/mL) 2%, high (50 ng/mL) 3%, patient sample 1%.

	Intra-assay (*n=6*)			Inter-assay (*n=6*)		
	Concentration (ng/mL): mean (SD)	Precision (% RSD)	Accuracy (%)	Concentration (ng/mL): mean (SD)	Precision (% RSD)	Accuracy (%)
LLOQ (0.2 ng/mL)	0.18 (0.02)	11.1	89.4	0.21 (0.07)	36.1	103.3
Low (1 ng/mL)	1.1 (0.09)	7.9	111.4	1.1 (0.08)	7.4	100.5
Mid (20 ng/mL)	22.8 (2.9)	12.9	114.1	23.2 (2.7)	11.8	104.3
High (50 ng/mL)	45.7 (3.8)	8.3	91.5	49.0 (1.8)	3.8	96.8
Patient sample	17.8 (0.97)	5.5		18.1 (1.0)	5.6	

Table 4-4 Summary table of precision and accuracy data, demonstrating acceptable intra-assay precision and accuracy to limits of 0.2 ng/mL. Inter-assay precision became acceptable at low point (1 ng/ mL) as defined U.S. FDA guidance (USFDA, 2001). SD, standard deviation; R, relative; LLOQ, lower limit of quantitation.

Initial run	After 24 hours in autosampler	After 28 days stored at -20 °C	Relative response
17.1 ng/mL	17.2 ng/mL		100.3%
17.9 ng/mL		16.8 ng/mL	93.9%

Table 4-5 Calculated concentration of tamsulosin in patient sample, demonstrating acceptable stability at 10 °C for 24 hours (in the autosampler) and at -20 °C for 28 days (in the freezer).

4.5 Method application

Analysis was performed as per the method described. For validation purposes the method was applied to samples from men who had received treatment (*n*=3), where concentrations of 16.7 – 36.1 ng/mL were quantified using 100 µL serum. These were within the quantitation limits of the assay. In serum obtained from men who had not received tamsulosin treatment (*n*=3), analyte was not detected (Figure 4.5). Results from clinical samples are presented in Chapter 5.4.3.2.

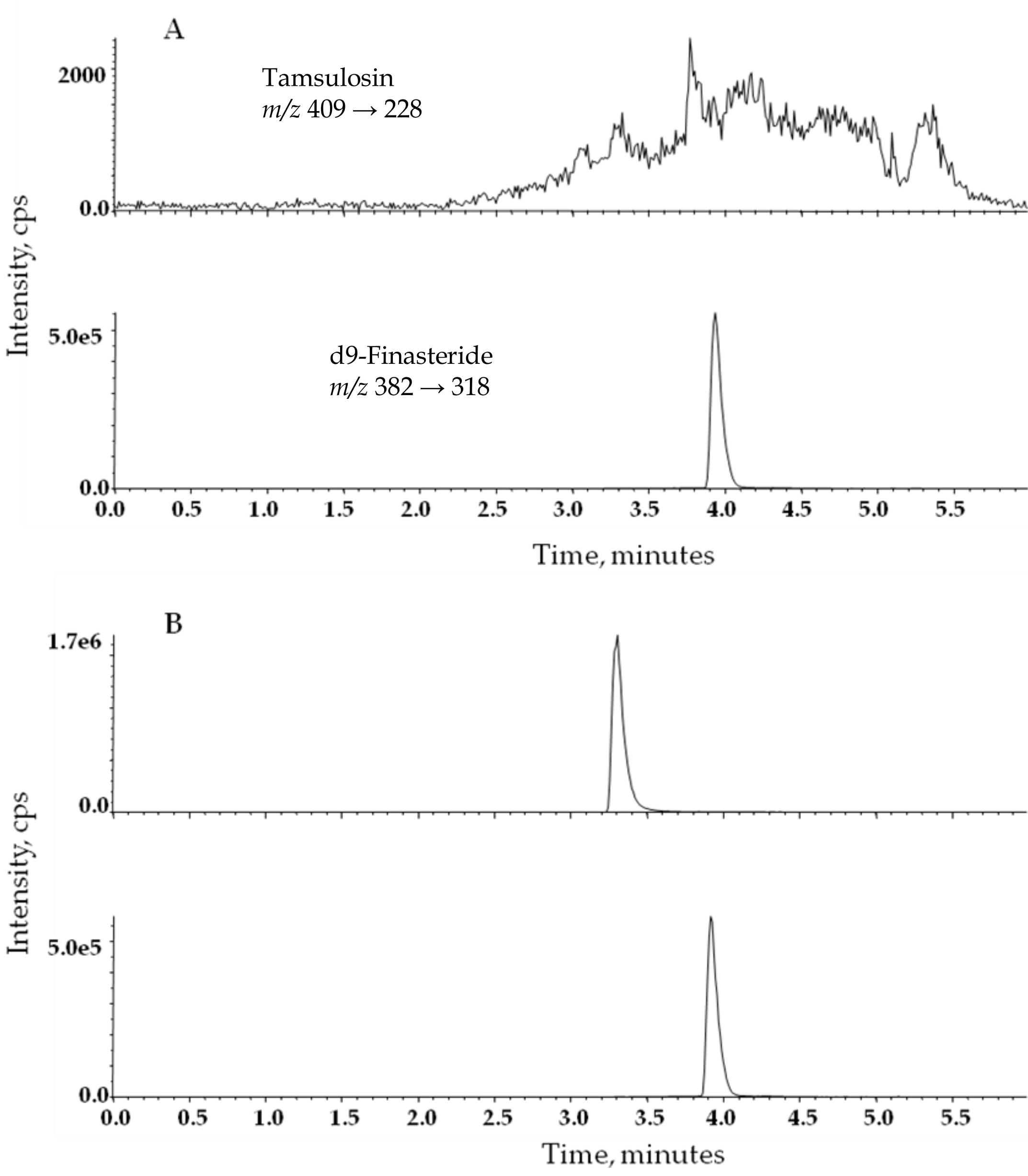

Figure 4.5 Application of method to serum from patient enriched with internal standard, d9-finasteride (10 ng/mL). For validation, method was applied to serum from patients on tamsulosin (*n=3*), and patients not on tamsulosin (*n=3*) with representative chromatograms shown. A. Demonstrates tamsulosin was not detected in serum from a patient not receiving tamsulosin. B. Demonstrates tamsulosin was detected in serum from a patient on treatment with 0.4 mg monitored released tamsulosin daily for 90 days, corresponding to a concentration of 26.2 ng/mL. cps, counts per second.

4.6 Discussion

The method developed is suitable for use for measurement of tamsulosin from human serum, and, in comparison to many other described methods, offers distinct advantages in terms of sample volume required (100 µL), limit of detection (0.13 ng/mL), ease of sample preparation and excellent analyte recovery (99.9%), a combination that has been missing from previous methods (Table 4-1).

One of the challenges faced during development was achieving symmetrical chromatographic peaks, a common problem with basic analytes such as tamsulosin, which interact by ion-exchange with unprotected silanol groups. Tamsulosin has a secondary amine group with a pKa of 8.4 and problems with peak tailing has been encountered by other researchers (Ding et al., 2002), typically requiring modification of the pH or buffering of the mobile phase. The addition of FA as a mobile phase modifier has been reported to be helpful (Ramakrishna et al., 2005a), aimed at reducing the pH less than 3 to ensure protonation of the silanol groups. However on our system basic rather than acidic modifiers were found to give a larger peak area for tamsulosin, while acidic modifiers yielded poor peak shape and impeded selectivity and resolution.

Before optimising the mobile phase, stationary phases with differing selectivity were compared, including PFP and C18 phase columns. PFP stationary phase can be useful with analytes containing aromatic rings, such as tamsulosin, given their ability to interact by π-π or dipole-dipole interactions. However with tamsulosin, the C18 phase provided the most consistent intensity of peak area and the most symmetrical peak shape when compared to other columns. A consistent finding was that alteration of gradient did not significantly improve the extent of peak tailing but, rather it was selection of the column that made the greatest difference. Overall, of the columns tested, the Ascentis Express demonstrated the best peak shapes and resolution, possibly reflecting the nature of its end-capping and was taken forward for further method development. Column temperature was optimised to 40 °C, seen as an improvement in peak shape.

Using the Ascentis column, trials of several aqueous mobile phases demonstrated the superiority of buffering with ammonium acetate in peak shape and area of both analyte and internal standard. This achieved a pH of 7.38 at which approx. 90% of tamsulosin would be ionised, enhancing the intensity of mass spectrometric response. Varying concentrations of ammonium acetate showed 2 mM (pH 7.38) to be the optimum in terms of analyte peak area, while still retaining consistency in chromatographic response. Higher concentrations of ammonium acetate (achieving a pH of ≈

6.6-6.9 at which tamsulosin would be expected to be close to 100% ionised) instead worsened peak areas, possibly due to the formation of ammonium adducts and associated ion suppression. Inconsistency in retention time was seen with lower (1 mM, 0.5 mM) concentrations, suggesting this was insufficient buffering capacity to overcome the silanol interactions. The addition of the stronger base triethylamine (TEA) has been reported to reduce peak tailing (Ding et al., 2002), the principle for this approach being that the strong base competes with the more weakly basic analyte for interactions with silanols. However when it was tested as a modifier on our system this did not improve peak tailing.

Under the final conditions selected, a total run-time of 6 minutes allowed high sample throughput. While shorter run-times of 2-3 minutes are described with isocratic methods (Matsushima et al., 1997, Ramakrishna et al., 2005a, Qi et al., 2004), when attempted on our system, these isocratic methods resulted in broad and tailing peaks, with decreased selectivity these (Schellinger and Carr, 2006). A shorter run time with gradient elution was not possible due to incomplete column re-equilibration.

Extraction methods published largely utilise traditional solid-phase (SPE) and liquid-liquid extraction (LLE) processes. Several extraction methods were compared during method development including LLE, supported

liquid, and SPE. Without modifying the pH of samples prior to extraction there was unacceptably high variability in replicate sample response with all approaches, probably a reflection of tamsulosin being partially ionised at neutral pH. As in other publications, a notable feature is the need for pre-extraction pH modification with basic modifiers such as $NaHCO_3$ (Ding et al., 2002, Matsushima et al., 1997), Na_2CO_3 (Qi et al., 2004, Macek et al., 2004) or NaOH (Ramakrishna et al., 2005a). LLE was taken forward with pH modification. A number of basic (ammonium acetate, ammonium hydroxide, sodium bicarbonate, sodium hydroxide) modifiers were tested in order to stabilise analyte extraction efficiency via LLE; the strongest base tested, with a pH of 10.9, ammonium hydroxide provided the best response in terms of peak areas and most consistent response with acceptable RSDs between replicate samples for both analyte and internal standard. Several extracting solvents were compared including ethyl acetate and hexane which gave poor and inconsistent recovery, diethyl ether and dichloromethane which gave consistent but very poor recovery. Following method optimisation (solvent type, proportions, multiples of extraction), excellent recovery and consistent responses were achieved with a liquid-liquid extraction with 20 volumes MTBE, to 1 volume sample, following mixing with 0.1 M ammonium hydroxide solution.

The ideal internal standard behaves in a similar fashion to the analyte throughout the extraction and analytical process. In the absence of a commercially available deuterated internal standard, structurally similar compounds (trichlormethiazide, ketoconazole and phthalylsulfathiazole) were explored as potential internal standards. Trichlormethiazide quantitation has been described with tamsulosin as internal standard due to structural similarities (Ramakrishna et al., 2005b), and both ketoconazole and phthalylsulfathiazole also share structural similarities with tamsulosin. However, while extraction efficiency of trichlormethiazide was reproducible, extraction of ketoconazole and phthalylsulfathiazole required pre-extraction pH modification that differed to conditions suitable for tamsulosin. In addition, none of these compounds behaved in a manner similar to tamsulosin in the mass spectrometer with very poor ionisation efficiency in positive ESI mode. Indeed most potential internal standards tested were well suited to ionisation in negative mode; however negative ionisation efficiency of tamsulosin was unsuitable for expected clinically-relevant concentrations. While switching between positive and negative ionisation modes is possible, this approach was not pursued. The internal standard ultimately selected was deuterated finasteride, which had been synthesised for use in the assay described in Chapter 3. While lacking in structural similarities to tamsulosin, d9-finasteride was well suited as an internal standard due to its very similar behaviour throughout the analytical process including extraction, ionisation

and detection. As tamsulosin is primarily used in treatment of BPH, where it is frequently co-prescribed with finasteride, it was important not to use unlabelled finasteride as internal standard. Analyte and internal standard are well resolved by chromatography, separated by mass, and the use of both quantifier and qualifier ions added further confirmation of specificity. While not optimised in the method described, due to its suitability for use with d9-finasteride, results suggest this method may be easily adapted to also measure finasteride. This would be particularly useful in other clinical studies in BPH where finasteride and tamsulosin are often used in different treatment groups, or administered as combination therapy.

Analyte specificity was ensured through use of LC-MS/MS analytical technique, together with monitoring of both quantifier and qualifier mass transitions. Metabolites of tamsulosin have different molecular weights to tamsulosin (Taguchi et al., 1997), and would therefore generate different precursor ions and mass transitions from their parent drug.

The method developed was suitable for measurement of tamsulosin from human serum, and, in comparison to many other described methods, offers distinct advantages in terms of sample volume required (100 μL), limit of detection (0.13 ng/mL), ease of sample preparation and excellent analyte recovery (99.9%), a combination that has been missing from previous

methods. The method was validated and conformed to internationally accepted standards. Expected concentration of tamsulosin fell within the linear range of the standard curve. Intra- and inter-day precision and accuracy were acceptable and stability testing demonstrated the assay to be applicable to normal laboratory practice. The described assay was successfully applied to samples taken from research volunteers taking tamsulosin.

Chapter 5: The effects of inhibition of 5α-reductases on metabolic health in men

5.1 Introduction

5αRs catalyse reduction of a range of steroid hormones, including androgens, glucocorticoids, progesterones and mineralocorticoids (Russell and Wilson, 1994). Metabolites are either more or less active than their parent steroid, and in some cases the activity of the metabolite remains uncertain. The best understood pathway of 5αR metabolism is the conversion of the androgen testosterone to its more potent metabolite, DHT. Inhibition of this pathway with 5αR inhibitors could conceivably have detrimental effects due to a decrease in DHT in tissues such as liver and adipose. A decrease in androgen action in liver, modeled through liver-specific genetic disruption of AR in male mice, results in hepatic steatosis and insulin resistance following a high-fat diet (Lin et al., 2008). Similarly, in adipose, male mice with fat-specific AR knockdown develop insulin resistance and later insulin deficiency and, when high-fat fed, this is further compounded by a susceptibility to increased visceral adiposity (McInnes et al., 2012).

In addition to effects on androgen metabolism, 5αR inhibition may also adversely affect metabolic health by alterations in glucocorticoid metabolism. Global attenuation of tissue glucocorticoid regeneration is protective to metabolic health, as demonstrated following transgenic disruption of 11βHSD1 in murine models (Kotelevtsev et al., 1997, Morton et al., 2004) and use of 11βHSD1 inhibitors in humans (Rosenstock et al., 2010). In addition,

tissue specific elevation in glucocorticoid concentrations in liver and adipose tissue are associated with features of the metabolic syndrome (Paterson et al., 2004, Masuzaki et al., 2001). One of the cortisol inactivating enzymes, 11βHSD2, when absent or deficient (Wilson et al., 2001), when inhibited by liquorice (Stewart et al., 1987), or in knockout mouse models (Kotelevtsev et al., 1999) shows predisposition to cortisol-induced hypertension. Overexpression of adipose 11βHSD2 in a murine model is protective against the metabolic syndrome (Kershaw et al., 2005). Upon this background, it follows therefore that other enzymes, such as 5αRs, regulating glucocorticoid concentrations in tissues including liver or adipose may also influence metabolic health. Specifically, impaired inactivation of glucocorticoids by 5αRs may induce accumulation of glucocorticoids in tissues with adverse metabolic consequences.

Preclinical data support this concept. In contrast to humans where both isozymes are expressed in liver, rats have only 5αR1 expressed in hepatic tissues, and mice express predominantly 5αR1 (Table 1-1). Male mice null for 5αR1 (5αR -/-; (Mahendroo et al., 1996)), when fed a 'western' (high fat, high sugar) diet developed obesity and hepatic steatosis, in addition to both fasting and post-glucose hyperinsulinaemia (Livingstone et al., 2008). A study in obese Zucker rats treated with finasteride (which, in contrast to humans, inhibits both 5αR isozymes in the rat (Azzolina et al., 1997))

demonstrated a propensity towards fatty liver and impaired glucose tolerance (Livingstone et al., 2009a). However there is a dearth of studies of metabolism in man following 5αR inhibition, with existing reports restricted to measures of fasting glucose (Amory et al., 2007). A key limitation in literature to date was the lack of a randomised trial in humans with detailed dynamic assessment of insulin sensitivity in response to 5αR inhibition.

Inhibitors of 5αR have been developed for the treatment of disorders such as benign prostatic hyperplasia, where reduced conversion from testosterone to the more potent androgen DHT reduces prostate size and thus improves obstructive urinary symptoms. Finasteride inhibits 5αR2 alone, while dutasteride is a dual isozyme inhibitor. While not their therapeutic intent, drugs inhibiting 5αR could also have consequences for other steroid metabolic pathways, including glucocorticoids. Slower glucocorticoid metabolism could, in theory, lead to an elevation in intra-tissue cortisol concentrations at sites where these enzymes are expressed, and importantly for this thesis liver and adipose. Since elevation of intra-tissue cortisol concentrations in other contexts is associated with features of the metabolic syndrome, particularly dyslipidaemia, obesity and insulin resistance, it follows that pharmacological inhibition of 5αR in man may lead to clinically significant adverse metabolic effects.

Isozymes of 5αR differ in their tissue distribution and this also varies between species; broadly 5αR1 is primarily expressed in metabolic tissues such as liver and 5αR2 is primarily expressed in reproductive tissues such as prostate (Table 1-1), with some 5αR2 also expressed in human liver. Given the observed differences in tissue distribution, it was hypothesised that metabolic dysfunction would be observed to a greater degree following inhibition of 5αR1 rather than 5αR2. In practice, since sole 5αR1 inhibitors are not available, the adverse effects might be manifest to a greater degree when both isozymes of 5αR are inhibited (with dutasteride), compared to inhibition of 5αR2 alone (with finasteride). Human 5αR2 deficiency is not described to have associated adverse metabolic consequences, however in the rare cases where a decrease in 5α reduction of cortisol was noted (Fisher et al., 1978), the metabolic consequences are not described. While a mouse model with genetic disruption of 5αR1 is widely used (Mahendroo et al., 1996), 5αR1 deficiency is not a known clinical entity in humans. Therefore pharmacological inhibition with dutasteride is currently the only means of investigating the consequences of 5αR1 inhibition in humans.

5.2 Hypothesis and Aims

5.2.1 Hypothesis

The principal hypothesis investigated was:

> Inhibition of 5α-reductases causes, or exacerbates, features of the metabolic syndrome, particularly insulin resistance. This effect may be mediated by increased glucocorticoid action in metabolic tissues.

An additional arm of the study investigated the effects of inhibition of 5αRs on HPA axis function. The background and hypothesis for this further work are presented in Chapter 6, but the study design is shown in its entirety in this chapter.

5.2.2 Aims

The aims of the study were to:

a) Establish whether pharmacological inhibition of 5αR in humans has an adverse effect on insulin sensitivity, resulting in alterations of glucose and lipid homeostasis.

b) Establish whether metabolic sequelae of 5αR inhibition are more pronounced with inhibition of 5αR1 and 5αR2 (with dutasteride), compared to inhibition of 5αR2 alone (with finasteride).

5.3 Methods

This section details the clinical methods used, initially describing the study design (Sections 5.3.1, 5.3.2, and 5.3.3), followed by each stage of the clinical study protocol (Section 5.3.4) and finally detailing clinical and analytical methods utilised in the study (Sections 5.3.5 to 5.3.8). Laboratory methods developed in support of this study are described in Chapters 3 and 4, and general laboratory methods used are described in Chapter 2.

5.3.1 Study design

Study design was that of a prospective, double-blind, randomised controlled study, with three treatment arms (dutasteride, finasteride, tamsulosin).

The study protocol included an initial screening visit (study day 'A'), an assessment of metabolism (study day 'B1') and an assessment of HPA axis function (study day 'C1'). Volunteers were then randomised to treatment with one of three arms – dutasteride (a dual 5αR1 and 5αR2 inhibitor), finasteride (a 5αR2 inhibitor) and tamsulosin (an α1 adrenergic antagonist, used as control group). Treatment was for 3 months, with standard doses used in therapy of benign prostatic hyperplasia, after which volunteers returned for a repeat assessment of metabolism (study day 'B2') and HPA

axis function (study day 'C2'), as well as an MRI scan for assessment of abdominal and hepatic fat.

An overview of the initial study design is presented in Figure 5.1.

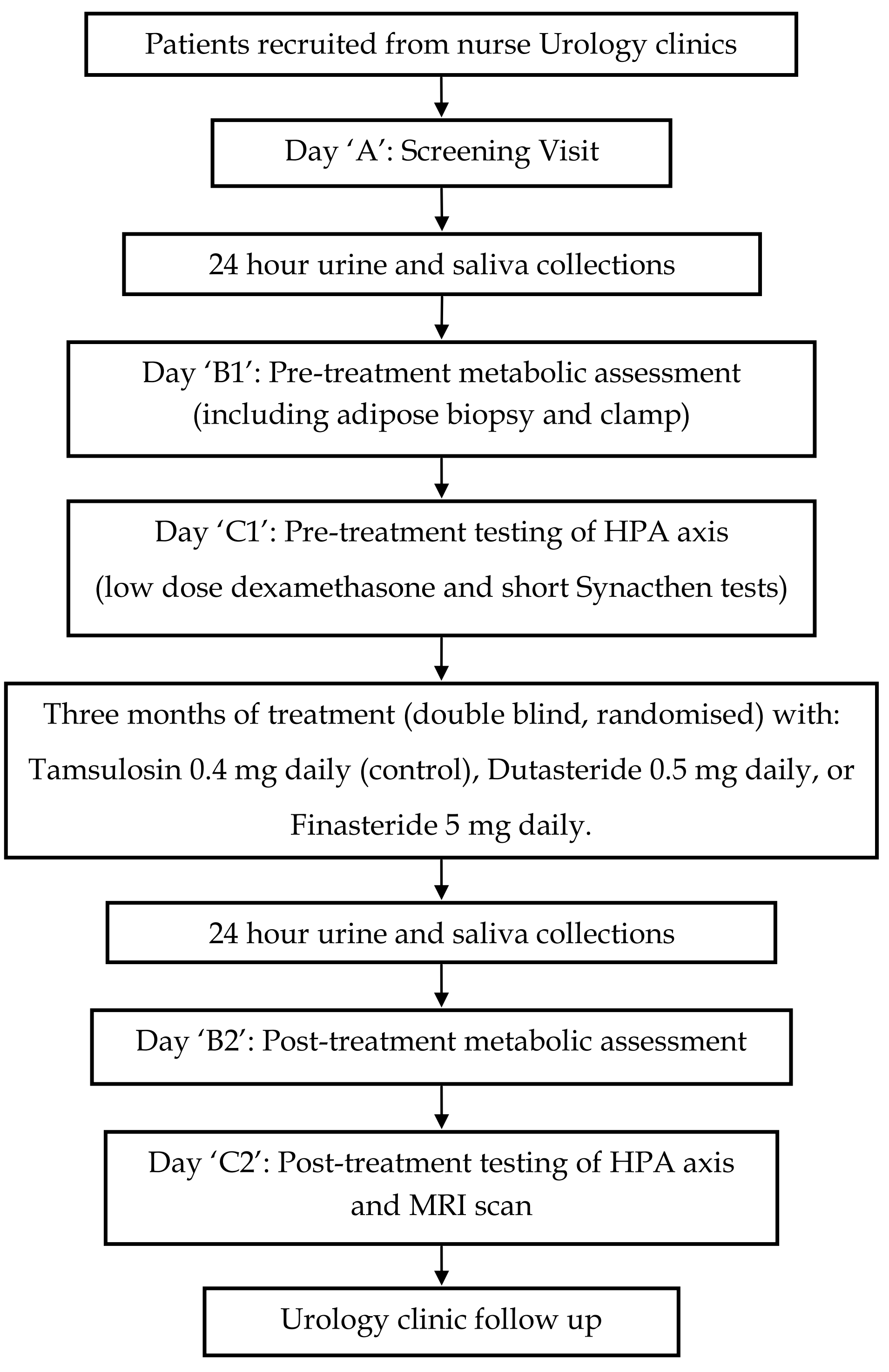

Figure 5.1 Summary of initial study protocol. HPA, hypothalamic pituitary adrenal; MRI, magnetic resonance imaging

5.3.2 Power calculations, sample size and interim analysis

This study was designed to enrol 99 men (33 in each arm of the study). Sample size calculations were performed using PS Power and Sample Size Program, version 3.0.14 (Dupont and Plummer, 1990). Ideally power calculations would be based on pilot data from a related study. However, a directly comparable study with paired population mean and variance data was unavailable at the time of planning, therefore calculations were based on mean (SD) glucose disposal rates during hyperinsulinaemia of 3.85 (0.88) mg/kg/min in a study 6 obese men treated with 7 days of carbenoxolone (a non-selective 11βHSD1 inhibitor) (Sandeep et al., 2005). The use of carbenoxolone did not alter insulin sensitivity in obese men studied. It was felt appropriate to use this study of obese men (rather than lean men) in the power calculations as the phenotype observed in animal models was in male mice on a high-fat diet with associated weight gain, therefore this was thought to be more comparable to the study planned. Using these data, to achieve 90% power to detect a 15% difference in glucose disposal rates to statistical significance (defined as $p<0.05$), a calculated sample size of 26 per group was required. A final group size of 33 per group was chosen, allowing for a > 20% dropout rate.

A single planned interim analysis was conducted when approximately half of the planned volunteers had completed participation (*n=38*), as the initial

power calculations had not been based on paired data and therefore had possibly over-estimated the sample size required. Data analysed at the interim analysis were M values during hyperinsulinaemia in baseline and post-treatment clamps, with a p value of <0.016 ($p<0.05$/ 3 groups) deemed in advance to be sufficient for stopping the study early. Interim results demonstrated a decrease in insulin sensitivity with dutasteride treatment compared to both finasteride and tamsulosin treated groups, with p values of 0.002 and 0.003 respectively. Based on these interim data, all volunteers enrolled in the study completed their participation, however no further recruitment took place and the study was stopped early. Data presented in this thesis are results from the final analysis only. There was no statistical adjustment made for the repeat assessment of the M value in the final analysis.

5.3.3 Ethical and Service Approvals for Study

The study was subject to peer review and supported by the Chief Scientist Office (CZB/4/642). Approvals from the Lothian Research Ethics Committee (09/S1101/33) and Research and Development (2009/W/END/01) office were obtained. Research support arrangements through the Western General Hospital (WGH) Urology service, Wellcome Trust Clinical Research Facility (E09732), laboratories at the WGH and the Royal Infirmary of Edinburgh (RIE), Scottish Brain Research Imaging Centre, RIE Radiopharmacy, WGH

Pharmacy, Tayside Pharmaceuticals (Dundee, UK) and the Scottish Primary Care Research Network (SPCRN) were obtained. The Medicines and Healthcare products Regulatory Agency (MHRA) confirmed that the study was not a Clinical Trial of an Investigational Medicinal Product, and therefore the study was conducted as a research study rather than a clinical trial, and did not have an assigned clinical trials number or a data monitoring committee. An internal independent advisor, Professor Jane E Norman (Centre for Reproductive Health, University of Edinburgh), provided informal advice during the implementation of the study. Dr Rebecca Reynolds (Endocrinology Unit, University of Edinburgh) was the internal independent contact for potential volunteers.

5.3.4 Study protocol

5.3.4.1 Inclusion and Exclusion Criteria

Inclusion criteria were men aged 50 - 80 years with symptomatic BPH requiring medical therapy. This was later widened to include all men aged ≥ 20 years (Study design and key amendments, Section 5.3.1), with healthy volunteers comprising the majority of participants studied, as presented in Section 5.4.4. Exclusion criteria were 5αR inhibitor use in previous 3 months, glucocorticoid use in previous 3 months, diabetes mellitus, impaired glucose tolerance, significant hepatic, renal or thyroid disease, hypogonadism,

warfarin therapy, body mass index ≥ 40 kg/m^2 or any suspicion of urological malignancy.

5.3.4.2 Recruitment

In the initial study protocol, patients with diagnosed BPH were recruited from Urology clinics at the Western General Hospital, Edinburgh. Eligible and interested patients were given a patient information sheet to read, then phoned at a convenient time. If interested, they were invited to attend a screening visit.

Due to difficulties with recruitment, the final study protocol was modified (Section 5.4.1) with the following key differences:

- Recruitment was extended to also include both primary care patients and healthy volunteers.
    - Primary care recruitment was conducted with the assistance of the Scottish Primary Care Research Network (SPCRN). SPCRN identified and recruited practices, and if consenting, searched primary care databases and generated a list of potential participants whose names were reviewed by the GP before invitations were sent out. The candidate was responsible for

gaining appropriate approvals, compiling recruitment packs and all follow up of patients who responded.

  - Healthy volunteers were recruited by advertising in newspapers, online and on poster boards around Edinburgh (principally at University of Edinburgh sites) and participation followed the same study protocol, though without any urological assessment or follow up.

- Age of eligibility was widened to include those ≥ 20 years.
- Study days C1 and C2 were made optional.
- Volunteer payments (in addition to travel expenses) were introduced from subject 6 onwards.

5.3.4.3 Study day A

Study day A, the screening visit, was scheduled at a convenient time of day and involved discussion of the study and review of the inclusion and exclusion criteria to ensure eligibility. If informed consent was obtained, participants were included in the study and a subject number was assigned at this stage. Clinical records were reviewed and, if not performed previously, a digital rectal examination was done (or organised through the patient's general practitioner). Height (Section 5.3.5.2), weight (Section 5.3.5.3), blood pressure and pulse (Section 5.3.5.1) were taken, along with

screening blood tests (full blood count, liver, kidney and thyroid function and random glucose, analysed as described in Chapter 2.14) collected by venepuncture from the antecubital fossa. Patients were asked to complete a 24 hour urine collection (Section 5.3.5.8) and saliva samples (waking, 30 minutes after waking, noon, 4pm, and bedtime) (Section 5.3.5.9) prior to the next study day.

5.3.4.4 Study day B

Study day B was designed to assess metabolism. Patients were asked to attend at 7:30 am, fasted from 10 pm the previous evening. Measurements for height, weight, systolic and diastolic blood pressure and pulse rate were repeated as per study day A. Hip and waist circumference were measured (Section 5.3.5.4) and bioimpedance measurements performed (Section 5.3.5.5). Basal fasting blood samples were taken (Section 5.3.5.6.1). Biopsies of subcutaneous abdominal adipose were taken (Section 5.3.5.10). A euglycaemic hyperinsulinaemic clamp was then carried out (Section 5.3.5.6).

5.3.4.5 Study day C

Study day C was designed to assess the HPA axis. The evening prior to the visit, patients were asked to take 250 μg (EC_{50} dose; (Reynolds et al., 2001)) dexamethasone (Essential Generics, Surrey, UK) orally at 10 pm and then remain fasted and attend at 7:30 am the following morning. A 20 gauge

cannula was inserted in the antecubital fossa and half an hour later a blood sample was taken. All sampling was from the inserted cannula (with the initial 2 mL of blood discarded) and blood was collected in lithium heparin tubes. Synacthen (1 µg; EC_{50} dose; (Daidoh et al., 1995)) was administered IV, and flushed with normal saline (0.9%, 10 mL). Further sampling was performed at 20, 30, 40 and 60 minutes. All samples were used for measurement of plasma cortisol (Chapter 2.6) and in addition baseline samples were also used for measurement of dexamethasone and dexamethasone metabolites (Chapter 2.13).

5.3.4.6 Treatment

Volunteers were randomised to double-blind once-daily treatment with one of tamsulosin MR (0.4 mg daily), dutasteride (0.5 mg daily) or finasteride (5 mg daily) for a period of 3 months (Section 5.3.6.1).

5.3.4.7 Reassessment post treatment including repeat study days B and C

At the end of the three month treatment period, participants were asked to return with a repeat 24 hour urine and saliva collection. Study days B and C were repeated in the same manner as pre-treatment tests. An MRI scan (Section 5.3.5.7) was performed in those without contra-indications to

scanning. Measurements were for intra-abdominal fat volume and proton spectroscopy to assess liver fat content.

5.3.4.8 Assessment of compliance with treatment allocation

Adequate compliance was deemed to be presence of drug in serum, measured by LC-MS/MS (Chapters 3 and 4). In addition, pills remaining were counted at the end of the study, and suppression of concentrations of 5α-reduced steroids in serum and urine (for the 5αR inhibitors) was assessed, however these were not regarded as defining compliance.

5.3.5 Clinical methods

5.3.5.1 Measurement of systolic and diastolic blood pressure and pulse rate

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, Hoofddorp, The Netherlands).

5.3.5.2 Measurement of standing height

Volunteers stood, without shoes, with feet parallel and head, shoulder blades and heels against the back of the wall mounted ruler. Measurement at the top of the head was recorded to the nearest 0.1 cm.

5.3.5.3 Measurement of weight

With outdoor clothing, shoes and heavy items from pockets removed, volunteer weight was measured in kilograms on an electronic scale (Seca, Birmingham, UK) after the instrument was calibrated.

5.3.5.4 Measurement of hip and waist circumference

Waist circumference was measured in a horizontal line, level with the umbilicus, with the volunteer standing with their hands by their sides, using a non-extensible measuring tape (Lufkin executive W606P). In the same standing position, hip circumference was measured by wrapping the tape measure around the buttocks and measurement taken at the point of maximum circumference. Measurements were made to the nearest 0.1 cm. Waist: hip ratio (WHR) was calculated as the waist circumference divided by the hip circumference.

5.3.5.5 Assessment of body fat

Body fat in mass and percentage was measured by electrical bioimpedance using an OMRON BF306 Body Fat Monitor (OMRON Healthcare (UK) Ltd, Henfield, UK) All measurements were conducted in the morning and following an overnight fast, though water intake was not regulated or recorded. The monitor was pre-set with the volunteer's height, weight, age

and gender. Manufacturer reported detectable range for body fat was 5 – 50%, and validated for use in ages 10 – 80 years. If aged > 80 years, bioimpedance was performed with age entered as 80 years and no adjustment to data was made for this. Electrodes were hand-to-hand, with weak alternating current (50 kHz, 0.5 mA) applied. Volunteers stood with their feet apart and arms held out at 90º, with the palm of each hand placed firmly on both electrode handles with fingers wrapped around the handles. Readings for body fat in kg and % were given by the monitor and results were taken as the average of three consecutive readings.

5.3.5.6 Hyperinsulinaemic euglycaemic clamp with deuterated glucose and glycerol tracers

A hyperinsulinaemic euglycaemic clamp was conducted for assessment of insulin sensitivity (method summarised in Figure 5.2). Three cannulae were inserted – two for venous infusions and one contra-lateral hand vein cannula inserted retrogradely for sampling arterialised blood during the clamp. The hand veins were 'arterialised' by external heating (Dreamland, Wirral, UK) throughout the clamp. The initial phase of the clamp was administration (Braun Infusomat® Space pump) of stable isotope tracers (Section 5.3.6.3). An initial priming bolus (over 1 minute) was administered (d2-glucose 17 μmol/kg; d5-glycerol 1.6 μmol/kg) to decrease time required to reach steady state. A tracer infusion was then commenced (d2-glucose 0.22 μmol/kg/min;

d5-glycerol 0.11 μmol/kg/min) and continued throughout the clamp. The second phase (90 - 180 minutes) consisted of low-dose Actrapid® insulin infusion (10 $mU/m^2/min$ - for inhibition of lipolysis and assessment of hepatic glucose output), with parallel 20% dextrose infusion adjusted to maintain euglycaemia. The third phase (180 - 270 minutes) consisted of high-dose Actrapid® insulin infusion (40 $mU/m^2/min$ – for assessment of peripheral glucose uptake), again with 20% dextrose infusion adjusted to maintain euglycaemia. Four steady state plasma samples were taken over a 20 minute period at the end of each phase. Glucose levels were clamped throughout at 4.5 - 5.5 mmol/L, as measured in whole blood from arterialised sample by glucometer (Accu-Check® Advantage, Roche, Germany).

5.3.5.6.1 Blood sampling during study day B

Sampling of fasting baseline blood was immediately following insertion of a 20 gauge anterograde intravenous cannula, subsequently used for infusions. Samples were used for measurement of: glucose (Chapter 2.14), insulin (Chapter 2.14), C-peptide (Chapter 2.14), androgens and 5αR inhibitor drugs (Chapter 3), tamsulosin (Chapter 4), DHEAS (Chapter 2.5), cortisol (Chapter 2.6), CBG (Chapter 2.7), adipokines (Chapter 2.9), SHBG (Chapter 2.14), and oestradiol (Chapter 2.14).

To obtain samples from arterialised hand vein cannula during clamps, the saline infusion keeping the cannula patent was discontinued, approximately 2 mL of blood/saline was removed and discarded, and the sample was then taken before the infusion of saline was resumed. Arterialised plasma samples were used for measurement of: glucose levels by glucometer during clamp (Section 5.3.5.6), and for later measurement of d2-glucose, glycerol and d5-glycerol (Chapter 2.11), insulin (Chapter 2.3) and NEFAs (Chapter 2.8).

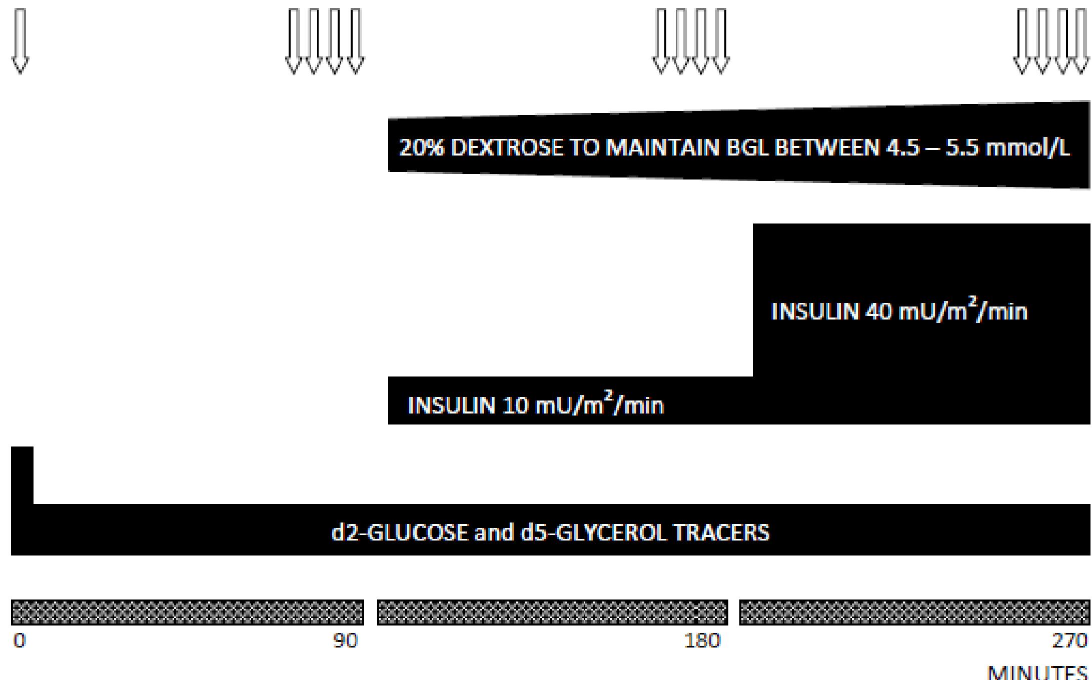

Figure 5.2 Schematic of hyperinsulinaemic euglycaemic clamp, consisting of three 90 minute phases. Deuterated tracers were given throughout clamp, with insulin administered in second (low dose insulin) and third (high dose insulin) phases of the clamp. 20% Dextrose was co-administered with insulin to maintain euglycaemia. Arrows represent sampling timepoints. BGL, blood glucose level.

5.3.5.7 Magnetic resonance imaging

Volunteers without contra-indications to scanning underwent magnetic resonance imaging (MRI) for intra-abdominal visceral and subcutaneous fat volumes, proton spectroscopy for liver fat measurements and structural images for clinical reporting. Scans were performed on a GE Signa Horizon 1.5T HDxt clinical scanner (General Electric, Milwaukee, USA) equipped with a self-shielding gradient set (33 mT m-1 maximum gradient strength) and manufacturer supplied Torso Array coil.

5.3.5.7.1 Visceral and subcutaneous abdominal adipose

Images for abdominal visceral and subcutaneous fat volumes were acquired in 10 mm slices with Iterative Decomposition of water and fat with Echo Asymmetry and Least-squares estimation (IDEAL) sequence, an imaging application designed for optimised fat quantitation.

Acquired samples were anonymised prior to interpretation. Measurements for abdominal fat were made with sliceOmatic version 4.3 (TomoVision, Magog, Quebec, Canada) software. Areas identified as either subcutaneous or visceral were identified by colour coding, and quantified with the programme software. All scans were analysed in 5 slices, with the central slice located at the L4/5 level. For comparison, data were analysed as both single slices at L4/5 and in 5-slice groups centred at L4/5. There was a

maximum difference of 2.7% in measured fat content and the two measures did not differ significantly, therefore single slice measures were utilised in data analysis.

Volume of adipose was expressed as estimated mass by taking into account the density of adipose tissue. The density assumed for results presented was 0.92 g/mL.

Intra-rater reproducibility was assessed by repeated (*n*=2, different days) measurement of all parameters on 6 scans, independently anonymised and randomly selected. Average coefficient of variation was calculated (standard deviation /mean * 100), and accepted if < 10% for each of visceral fat, subcutaneous fat, or abdominal volume. Acceptable results were obtained with mean CV of 6.9% for visceral fat, 2.2% for subcutaneous fat and 0.3% for abdominal volume.

5.3.5.7.2 Liver ^{1}H magnetic resonance spectroscopy

Single voxel proton MR spectroscopy was performed for assessment of hepatic fat, using the body coil of the same scanner, using a point-resolved spectroscopy sequence (PRESS), with water suppression. The voxel was positioned using structural T2 images obtained during breath hold (inspiration). Voxel size was 10 mm^3 and was demarcated by 6 saturation

bands (anterior, posterior, superior, inferior, left and right) reviewed in axial, coronal and sagittal planes. The voxel was positioned within the liver, avoiding the edge of the liver and major vessels. An example of voxel placement is shown in Figure 5.3. Spectra were acquired during free breathing, with echo time (TE) 40 ms and relaxation time (TR) 5000 ms.

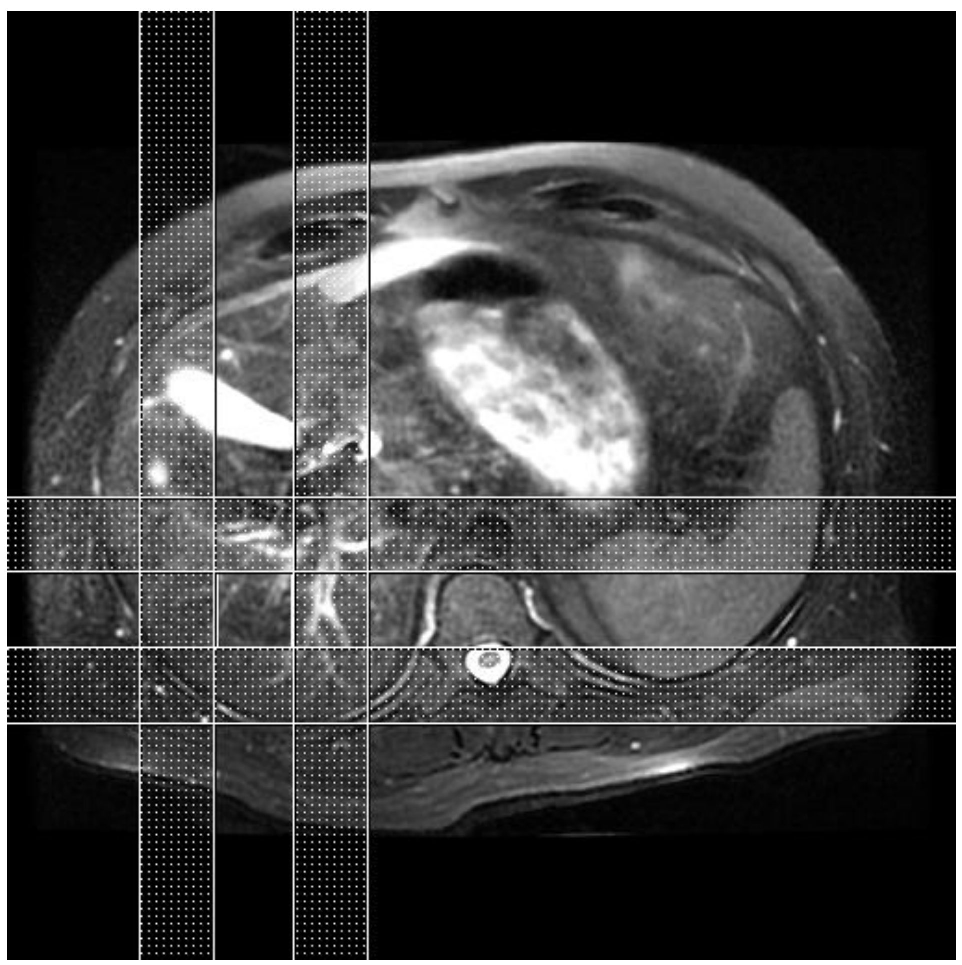

Figure 5.3 Representative axial image of voxel placement for MR spectroscopy, utilised for quantitation of fat in human liver. The voxel was 10 mm^3 in size and was demarcated by saturation bands reviewed in axial, coronal and sagittal planes. The voxel was positioned over the liver in an area as devoid of vascular structures as possible.

Post-processing and quantification of MRS data was performed in jMRUI (Naressi et al., 2001a, Naressi et al., 2001b) using a nonlinear least-squares algorithm (AMARES, (Vanhamme et al., 1997)) with Gaussian line shapes to model each spectral peak of interest (e.g., water at 4.7 ppm, methylene fat at 1.3 ppm). Fat fraction was subsequently defined as the ratio of the area under the fat peak to the combined areas under the water and fat peaks:

$$Fat\ fraction(\mathrm{FF})_{MRS} = \frac{area\ under\ fat\ peaks}{(area\ under\ fat\ and\ water\ peaks)} \times\ 100\%$$

Scans with a large fat fraction (> 20%) had all data manually reviewed and confirmed as technically reliable prior to inclusion.

5.3.5.7.3 Images for clinical reporting

To comply with ethical requirements, additional structural images (T2 liver) were acquired for clinical reporting by a radiologist checking for incidental findings, and all results were conveyed to the patient's general practitioner. These images were not analysed as part of the study.

5.3.5.8 Urine collection

Volunteers were asked to collect 24 hour urine collections at home. Urine was collected from the second void after waking for a 24 hour period in a non-acidified collection bottle. Total volume was recorded and samples were

processed and stored as described in Section 5.3.7. Samples were used for measurement of urinary androgens and glucocorticoids, as described in Chapter 2.12.

5.3.5.9 Saliva collection

Volunteers were asked to complete a collection of 5 saliva samples during the day (waking, 30 minutes after waking, noon, 4 pm, and bedtime) using Salivette® collection tubes (Sarstedt, Sarstedt, Germany). Collections were separated from meals and other potential contaminants (e.g. toothpaste, cigarettes). Samples were processed and stored as described in Section 5.3.7. Samples were used for measurement of salivary cortisol, as described in Chapter 2.4.

5.3.5.10 Biopsy of subcutaneous abdominal fat

Local anaesthetic (2% Lignocaine, Hameln Pharmaceuticals, Gloucester, UK) was injected subcutaneously at a site lateral to, and directed towards, the umbilicus. A 14 gauge needle attached to a 30 mL syringe was inserted into subcutaneous adipose, the plunger of the syringe withdrawn to create a vacuum then held in place with a test tube (12 x 75 mm, Fisherbrand®, Fisher Scientific, Loughborough, UK) while a sample of adipose was obtained. Samples were washed (through Isopon™ aluminium mesh, DF Wishart, Edinburgh, UK) with DEPC water, then frozen immediately on dry ice before

storage (-80 ºC) for future analysis. Three samples were obtained in the same manner. Fat biopsies were not collected if felt to be unsafe (due to very low amounts of subcutaneous abdominal fat). Samples were used for analysis of mRNA abundance of genes of interest (Chapter 2.10).

5.3.6 Study medication and intravenous drug preparations

5.3.6.1 Study medication randomisation and dispensing

Volunteers were randomised to double-blind once-daily treatment with one of tamsulosin MR (0.4 mg daily, Synthon Hispania, Sant Boi de Llobregat, Spain), dutasteride (0.5 mg daily; Glaxo Smith Kline Pharmaceuticals (Poznań, Poland)) or finasteride (5 mg daily; Gedeon Richter (Budapest, Hungary)). Medication was dispensed in one bottle containing 100 capsules.

Randomisation was performed independently of the investigation team, by Tayside Pharmaceuticals (Dundee, UK) through a computerised random number generator (http://www.randomization.com; reference seed 25371; created 09 December 2009). Randomisation method was fixed–size block randomisation (*n=18* per block). No stratification or minimisation was used in the randomisation process. Allocation concealment was ensured by appropriate storage of the randomisation code (sealed envelope in locked

storage) so neither study personnel conducting study visits, nor study participants, could predict group assignment prior to study enrolment.

The study was double-blind, with both investigator and participant blinded to treatment allocation throughout the study period. All medications were blinded (by enclosing in a hard gelatin capsule and packing with Lactose Ph Eur) and stored in numbered but otherwise identical bottles. Monthly contact was made with volunteers, and they were given a wallet-sized card with routine contact details for the candidate and emergency contact details for on-call pharmacy.

Patient volunteers were unblinded at the end of the study period (at the earliest this was *after* the clamp on visit 'B2') to allow decisions regarding ongoing care. Healthy volunteers were unblinded at either the interim or final analysis.

5.3.6.2 Sodium chloride (0.9%)

Sodium chloride (0.9%) (Baxter, Thetford, Norfolk, UK) was used as a diluent for infusions and flush for cannulae.

5.3.6.3 Stable isotope tracers: d2-glucose and d5-glycerol

Deuterated tracers were from Cambridge Isotope Laboratories, Inc. (Andover, USA). Chemical and isotopic purity were assessed by the manufacturer, and tracers were certified to be manufactured via a chemical route and therefore without risk of Transmissible Spongiform Encephalopathies.

a) 6, 6- $[^2H]_2$-glucose (d2-glucose): ≥ 98% purity as determined by HPLC and ≥ 99% isotopic enrichment as determined by GC-MS.
b) 1, 1, 2, 3, 3 – $[^2H]_5$-glycerol (d5-glycerol): ≥ 98% purity as determined by HPLC and ≥ 99% isotopic enrichment as determined by GC-MS.

Tracers were prepared in sterile solutions by the Radiopharmacy (Royal Infirmary of Edinburgh, Edinburgh, UK), as follows:

a) d2-glucose: 2.11 g / 6 mL, to give concentration of 350 mg/mL
b) d5-glycerol: 380 mg/ 9.5 mL, to give concentration of 40 mg/mL

Solutions were stored (-40 °C) for a maximum of 8 weeks.

Solutions were defrosted and infusions prepared in the clinical research facility on the morning of each study.

Drug for infusion:

d5-Glycerol (7.5 mL, 300 mg) was added to sodium chloride (0.9%, 489.3 mL) to give a volume of 496.8 mL. d2-Glucose (3.2 mL, 1.12 g) was added to make up to total of 500 mL. Final concentrations were: d5-glycerol 300 mg/ 500 mL and d2-glucose 1.12 g/ 500 mL. Infusion was administered as described in Section 5.3.5.6 .

Drug for bolus:

d5-Glycerol (0.5 mL, 20 mg) was added to sodium chloride (0.9%, 19.5 mL) to give a concentration of 1 mg/mL. d2-Glucose (1 mL, 350 mg) was added to sodium chloride (0.9%, 19 mL) to give a concentration of 17.5 mg/mL. Boluses were administered prior to infusion as described in Section 5.3.5.6.

5.3.6.4 Insulin

The infusion was prepared from 100 IU/ mL vial of Actrapid® insulin (Actrapid®, Novo Nordisk, West Sussex, UK). Actrapid (0.3 mL, 30 IU) was added to 0.9% sodium chloride (100 mL) to give a final concentration of 0.3 IU/mL, and was then infused as described in Section 5.3.5.6.

5.3.6.5 20% Dextrose

Dextrose infusion was dextrose (20%, *w/v*) (Baxter, Thetford, Norfolk, UK) containing 200 g anhydrous dextrose per 1000 mL water.

5.3.6.6 Synacthen

Bolus for intravenous administration was prepared from 250 µg/mL vial of Tetracosactide (Alliance Pharmaceuticals Ltd., Chippenham, Wiltshire, UK; Tetracosactide is referred to as Synacthen throughout this thesis,). Synacthen (1 mL) was added to 0.9% sodium chloride (49 mL) to give a concentration of 5 µg/mL. Synacthen (1 µg as 0.2 mL of 5 µg/mL) was added to 0.9% sodium chloride (0.8 mL) to give a final concentration of 1 µg/mL, and was administered immediately, as described in Section 5.3.4.5.

5.3.7 Sample collection tubes and sample processing

All blood tubes used were S-Monovette® tubes (serum gel 7.5 mL or 4.9 mL to collect serum, lithium heparin 5.5 mL or 2.7 mL to collect plasma, EDTA 2.7 mL to collect whole blood, fluoride gel 2.7 mL for glucose) from Sarstedt (Sarstedt, Germany).

Samples not sent directly to NHS Lothian laboratories were processed on-site at the WTCRF following collection as follows, and stored for future analysis.

Equipment used was:

a) Sigma 4K15 Centrifuge (Münich, Germany)

b) Micromail II scales (Salter Brecknell, Smethwick, UK)

All blood samples collected were prepared by centrifugation (1912 g, 10 minutes, 4 ºC) and collected plasma/ serum frozen (-80 ºC) for later analysis.

The volumes of the 24 hour urine collections were measured, and 6 aliquots (~20 mL each) frozen and stored (-40 ºC) for later measurement of glucocorticoid and androgen metabolites (Chapter 2.12).

Saliva was collected by centrifugation of the collection tubes (1912 g, 5 minutes, 4 ºC) and frozen (-40 ºC) until analysis at a later date for salivary glucocorticoid concentrations (Chapter 2.4).

5.3.8 Data analysis

5.3.8.1 Body mass index

Body mass index (BMI) was calculated as:

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

5.3.8.2 Body surface area

Body surface area (BSA) was calculated according to the Mosteller method (Mosteller, 1987) with the equation:

$$BSA\ (m^2) = \sqrt{\frac{height\ (cm) \times weight\ (kg)}{3600}}$$

5.3.8.3 Homeostatic model assessment of insulin resistance (HOMA-IR)

HOMA-IR was calculated based on the original description (Matthews et al., 1985) with the equation:

$$HOMA-IR = \frac{fasting\ glucose\ (mmol/L) \times fasting\ insulin\ (\mu U/mL)}{22.5}$$

where a higher value is indicative of lower insulin sensitivity.

5.3.8.4 Quantitative insulin sensitivity check index (QUICKI)

QUICKI was calculated as originally described (Katz et al., 2000) with the equation:

$$QUICKI = \left(\frac{1}{Log\ insulin(\mu U/mL)\ + Log\ glucose\ (mg/dL)}\right)$$

where a higher value is indicative of greater insulin sensitivity.

5.3.8.5 M value

In relation to the hyperinsulinaemic euglycaemic clamp, the mean steady state infusion rate of glucose (‘M value’) was calculated as the average infusion rate during the sampling period of 15 minutes at the end of the high dose insulin phase (+255 to +270 minutes) of the clamp.

5.3.8.6 Glucose and glycerol tracer kinetics

In order to calculate tracer kinetics during the hyperinsulinaemic euglycaemic clamp the following equations were used:

At steady state:

(Equation 1.2.6.7.1)

$$\frac{Rate\ of\ appearance\ (Ra)\ d2\ glucose}{Ra\ glucose} = \frac{peak\ area\ d2\ glucose}{peak\ area\ glucose}$$

And:

(Equation 1.2.6.7.2)

$$\frac{peak\ area\ d2\ glucose}{peak\ area\ glucose} = tracer\ tracee\ ratio\ (TTR)$$

Therefore:

(Equation 1.2.6.7.3)

$$Ra\ glucose\ = \frac{Ra\ d2\ glucose}{TTR}$$

This equation was used to calculate the endogenous production of glucose at baseline (without insulin infusion) during the first phase of the clamp.

However, during the next two phases of the clamp (low dose insulin, high dose insulin) glucose was also administered IV as part of the clamp (to maintain euglycaemia during insulin infusion), therefore:

(Equation 1.2.6.7.4)

$$Ra\ glucose\ = endogenous\ Ra\ glucose\ + infused\ Ra\ glucose$$

It follows that:

(Equation 1.2.6.7.5)

$$Endogenous\ Ra\ glucose\ + Infused\ Ra\ glucose\ = \frac{Ra\ d2\ glucose}{TTR}$$

This was equivalent to the rate of disposal of glucose.

To calculate the endogenous production of glucose, the equation used was:

(Equation 1.2.6.7.6)

$$Endogenous\ glucose\ production\ = \left(\frac{Ra\ d2\ glucose}{TTR}\right) - Infused\ Ra\ glucose$$

For both glucose and d2 glucose, the contribution of the mass + 0 isotope to the total amount is 92.5%, and the contribution of the mass + 2 isotope is 1.1%. Appropriate corrections were applied to adjust both the infused glucose and d2 glucose amounts.

To calculate the rate of appearance of glycerol the following equations were used:

At steady state:

(Equation 1.2.6.7.7)

$$\frac{Ra\ d5\ glycerol}{Ra\ glycerol} = \frac{peak\ area\ d5\ glycerol}{peak\ area\ glycerol}$$

And:

(Equation 1.2.6.7.8)

$$\frac{peak\ area\ d5\ glycerol}{peak\ area\ glycerol} = tracer\ tracee\ ratio\ (TTR)$$

Therefore, as glycerol was not being infused, the equation used was:

(Equation 1.2.6.7.9)

$$Ra\ glycerol = \frac{Ra\ d5\ glycerol}{TTR}$$

For both glucose and glycerol kinetics, equivalent ionisation of both tracer and tracee was ensured by monitoring of enrichment curves, therefore the ratio of peak areas was deemed a robust assessment.

5.3.8.7 Statistical methods

Statistical analysis was performed using SPSS for Windows software package, version 19 (IBM, Portsmouth, UK). Data analysed were of the 46 volunteers who completed and were deemed compliant with the study treatment protocol (as defined in Section 5.3.4.8). Data are presented as mean (standard deviation, SD) unless otherwise stated. Comparisons are presented as mean (95% confidence interval), together with test of statistical significance. ANalysis of COVAriance (ANCOVA) is usually considered the ideal statistical method for randomised studies with pre- and post-intervention measures (Vickers and Altman, 2001). In order to use an ANCOVA as the statistical approach, it is important that the ANCOVA model fits the data. The key assumptions which need to be met are: a normal distribution, linear regression (a linear relationship between the post-treatment and pre-treatment values), homogeneity of variance (similar scatter of individual data points around regression lines for each treatment group) and homogeneity of regression lines (similar, parallel, regression slopes for each treatment group). The latter (homogeneity of regression lines) is a key assumption in this model. In the present study while the ANCOVA was explored, it was not suitable for use, as the primary and many secondary endpoints in the study did not meet necessary assumptions, in particular regression lines were not parallel between treatment groups, invalidating this model. Expert statistical advice was received from Professor Gordon D

Murray (Centre for Population Health Sciences, University of Edinburgh) who advised that it would be inappropriate to pursue using an ANCOVA when the analytical assumptions are not met, and suggested changing of the statistical methodology to the simpler but still valid and robust method using analysis of change from baseline. While a repeated-measures ANOVA is used by some in such study designs, this was not felt to be appropriate as assumptions for this were not met including differences in group sizes, as well as a lack of sphericity (equal variance in the differences between treatment groups) in some endpoints. Analysis as change from baseline with post-hoc testing takes into account repeated measures and allows pairwise comparisons between groups.

The final statistical method selected for use and presented in this thesis was comparison between treatment groups by ANalysis Of VAriance (ANOVA) on the change from baseline in each treatment group, unless stated otherwise in the text. Where ANOVA was significant ($p < 0.05$), further post-hoc testing was performed with a Least Significant Difference (LSD) test to allow pairwise comparisons. Data was tested for normality of distribution by visual assessment of histograms. Homogeneity of variance was tested by Levene's test, and accepted if result was non-significant ($p > 0.05$). If needed and if appropriate, data transformation was tested by log, square root, square, and reciprocal, and the method selected was that which resulted in a normal

distribution. If assumptions were not met then non-parametric testing was used (Kruskal-Wallis test). For MRI scans, results were analysed as above though only for the post-treatment measure as there was no baseline measure.

Statistical significance was taken at the 5% level. Where values are missing these are indicated and not imputed. Nor was there additional adjustment made for multiple comparisons. For statistical analysis, values below the detection limit were considered to be one third of the limit of detection for the assay.

5.4 Results

5.4.1 Study design and key amendments

In the first 9 months recruitment into the study from Urology clinics was poor, with only 2 patients enrolled (plus two withdrawals on medical grounds, Section 5.4.3.3). Review of recruitment revealed this was largely due to low numbers of patients seen in clinic, and also the majority of patients being ineligible for, or declining participation for reasons including the time commitment involved in the study or a reluctance to change medication.

In order to address poor recruitment rates, during the next 7 months, a series of changes were made to the original protocol. Attempts were made to address concerns expressed by some patients with respect to time commitments required by making study day C optional, and potential loss of income due to attendance at study visits by introducing volunteer payments. Further efforts were made to improve access to Urology patients seen in clinics, and also extend recruitment to patients seen in primary care. Due to ongoing poor recruitment, recruitment of healthy volunteers was commenced.

5.4.2 Recruitment rates

Recruitment improved with the implementation of changes described above, though still lagged significantly behind the original targets. Recruitment from the Urology Clinic slowed further after 1 year when other avenues of recruitment were prioritised. In total, 39 primary care practices were invited, 3 practices agreed to assist, and a total of 37 patients were invited, of which 30 were suitable for inclusion, and 7 agreed to participate. Healthy volunteer recruitment was primarily via advertising and later also by word of mouth. Overall response and recruitment rates are summarised in Figure 5.4.

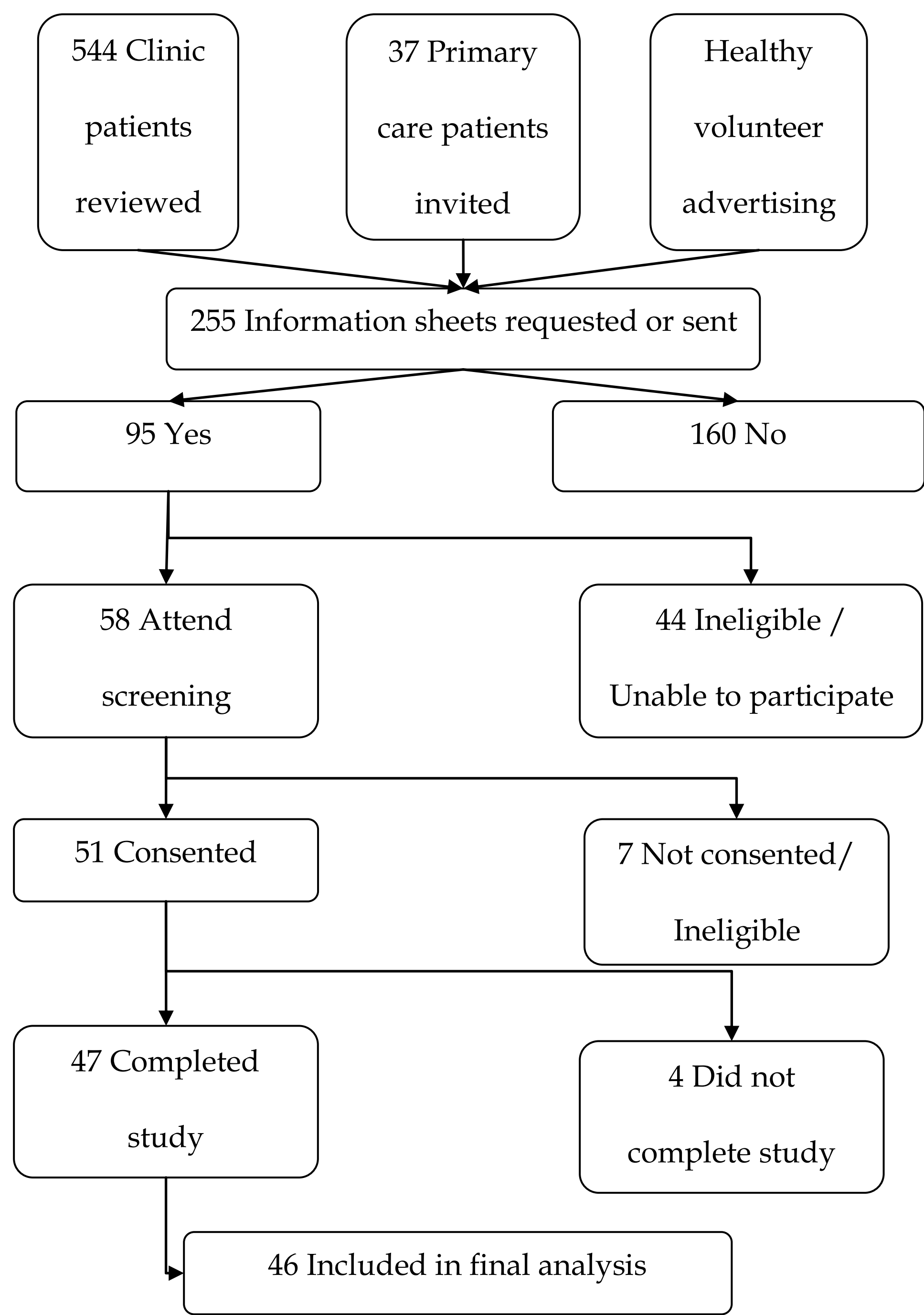

Figure 5.4 CONSORT diagram of overall recruitment rates. 'Yes' indicates an interest in participation, 'No' indicates either an unfavourable response or no response. Two of the 4 volunteers who did not complete the study were assigned a study number following informed consent but did not participate further. CONSORT, consolidated standards of reporting trials (Moher et al., 2012).

5.4.3 Compliance and study participation

5.4.3.1 Pill count

Pills missed (by pill count) were, on average, 4.1% (dutasteride), 3.7% (finasteride), and 3.2% (tamsulosin).

5.4.3.2 Serum drug concentrations

Dutasteride and finasteride were only detected in volunteers prescribed the medication, and neither was detected in those in the tamsulosin group. Dutasteride was not detected in serum of one subject in the dutasteride group, suggesting non-compliance with his assigned medication. In addition his serum DHT was not suppressed, nor his urinary steroids ratios (described for compliant subjects in Section 5.4.5) altered as expected with 5αR inhibitor treatment. This volunteer was therefore excluded from subsequent study analyses. Amongst other volunteers in the dutasteride group, serum levels detected were between 3.0 – 28.5 ng/mL, in line with expected concentrations (Chapter 3).

All subjects in the finasteride group had detectable drug in serum, in the range 2.0 – 64.0 ng/mL, in line with expected concentrations (Chapter 3).

Tamsulosin was not detected in any volunteers who were not prescribed the drug in the study but was detected in serum from the six patients who were receiving this drug prior to randomisation into the study (range 2.8 – 12.9 ng/mL). In samples taken at the end of the study, tamsulosin was detected in all patients who were in the tamsulosin group, and also the patient requiring rescue tamsulosin treatment (Section 5.4.3.3), with an average serum drug level of 8.3 ng/mL (range 1.7 – 15.2 ng/mL), in line with expected concentrations (Chapter 4).

5.4.3.3 Study participation

Of the 51 men who consented to participation, 4 did not complete the study. One volunteer was taken out of the study due to persistent subclinical hypothyroidism on biochemical screening, due to concerns this may impact on study outcomes. One volunteer receiving finasteride withdrew from the study due to the development of urinary retention and impotence early in the study period. A further two volunteers did not return for study visits following screening due to the development of unrelated illness and subsequent inability to negotiate further time away from study and employment. Of the 47 volunteers who completed the study, 46 were included in the final analysis. As detailed above (Section 5.4.3.2) one volunteer (in the dutasteride group) had no detectable dutasteride in serum, therefore was excluded from the analysis. Measurement of body fat was not

possible in one volunteer (in the dutasteride group), due to amount of body fat being below the sensitivity of the bioimpedance instrument (< 5% body fat).

One volunteer (from the finasteride group) did not attend for visits C1 or C2, therefore analysis of dynamic testing of the HPA axis was performed with 45 volunteers (Chapter 6).

One BPH patient developed intolerable symptoms upon cessation of his usual tamsulosin at the start of the study period; he was able to complete the study with partial unblinding and administration of unblinded tamsulosin MR 0.4 mg daily along with the blinded study medication (which was confirmed by the independent study advisor not to be tamsulosin in order to prevent over-medication). Adverse effects and outcomes are summarised in Table 5-1.

Volunteer type	Nature of adverse event (severity)	Outcome
Patient	Subclinical hypothyroidism on biochemical screening	Withdrawn
Patient	Urinary retention and impotence (severe)	Withdrew
Healthy	Dizziness and diarrhoea (mild)	Continued
Healthy	Diarrhoea (mild)	Discontinued tablets for 3 weeks, then recommenced with no further problems
Healthy	Impotence (mild)	Continued
Patient	Worsening of BPH symptoms (moderate)	Continued
Patient	Worsening of BPH symptoms (severe)	Continued with rescue tamsulosin
Patient	Recurrence of chronic leg pain (mild)	Discontinued tablets for 2 weeks, then recommenced with no further problems

Table 5-1 Summary of reported adverse events during study. The two subjects who temporarily discontinued tablets went on to complete at least 90 days of treatment before follow up visits were completed. Severity of symptoms is as reported by the volunteer, not as defined by the Good Clinical Practice International Clinical Trial Harmonisation directives.

The study protocol specified 3 months of medication prior to follow-up visits. The average and range of days in the study is summarised by group in Table 5-2. The individual in the dutasteride group who had 111 and 112 days at visits B2 and C2 respectively had intermittently missed pills through the study therefore leaving him with additional days on medication past the 100 pills given to each volunteer.

		Dutasteride	Finasteride	Tamsulosin
	N	*16*	*16*	*14*
Days at visit B2	**Average**	94.8	91.6	92.2
	Range	91 - 111	86 - 99	87 - 97
	N	*16*	*15*	*14*
Days at Visit C2	**Average**	97.6	93.5	95.1
	Range	93 - 112	87 - 101	89 - 100

Table 5-2 Length of time participants were on prescribed study medication, indicating average, minimum and maximum by group. N, number.

5.4.4 Volunteer characteristics at baseline

Volunteers were randomised to one of three treatment groups. Key characteristics of participants at baseline are summarised in Table 5-3.

	Dutasteride	Finasteride	Tamsulosin	Difference
N	*16*	*16*	*14*	
Age	35.3 (14.6)	40.3 (19.2)	49.4 (18.4)	p=0.12
BPH patients (*N*)	2	4	5	N/A
BMI (kg/m^2)	25.3 (4.41)	26.8 (3.82)	25.5 (2.76)	p=0.49
WHR	0.90 (0.08)	0.89 (0.07)	0.93 (0.06)	p=0.39
SBP (mmHg)	131.0 (11.43)	136.4 (14.91)	138.8 (18.26)	p=0.35
DBP (mmHg)	77.6 (9.96)	78.4 (11.19)	81.3 (8.85)	p=0.58
Body fat (kg)	16.5 (8.70) *(N=15)*	18.9 (6.97)	20.1 (5.94)	p=0.40
Body fat (%)	19.8 (8.49) *(N=15)*	22.1 (6.62)	24.7 (5.99)	p=0.19
Glucose (mmol/ L)	4.96 (0.49)	4.98 (0.50)	5.14 (0.37)	p=0.54
Insulin (pmol/L)	58.8 (23.79)	54.1 (17.85)	63.2 (32.97)	p=0.62
C-peptide (pmol/L)	539 (156.9) *(N=15)*	539.4 (172.9)	612.9 (295.7)	p=0.57
HOMA-IR	1.89 (0.79)	1.73 (0.63)	2.19 (1.14)	p=0.54
Total C (mmol/L)	4.41 (0.64)	4.87 (1.01)	5.20 (0.95)	p=0.06
TG$^+$ (mmol/L)	1.25 (0.6)	1.21 (0.7)	1.35 (0.7)	p=0.83$^+$

Table 5-3 Key characteristics of study participants at baseline. Data are mean (SD). Blood tests presented were all fasting samples. In a randomised study any differences at baseline are recognised to occur by chance. When assessed by one-way ANOVA there were no differences between groups at baseline. $^+$Raw data is presented, however was log transformed for statistical analysis. N, number; BMI, body mass index; WHR, waist: hip ratio; S, systolic; D, diastolic; BP, blood pressure; HOMA-IR, homeostatic model assessment of insulin resistance; C, cholesterol; TG, triglycerides.

With the inclusion of healthy volunteers all groups were, on average, younger than most BPH patients, however as an indicator of whole body 5αR activity at baseline, the serum DHT: T ratio did not correlate with age, as seen in Figure 5.5.

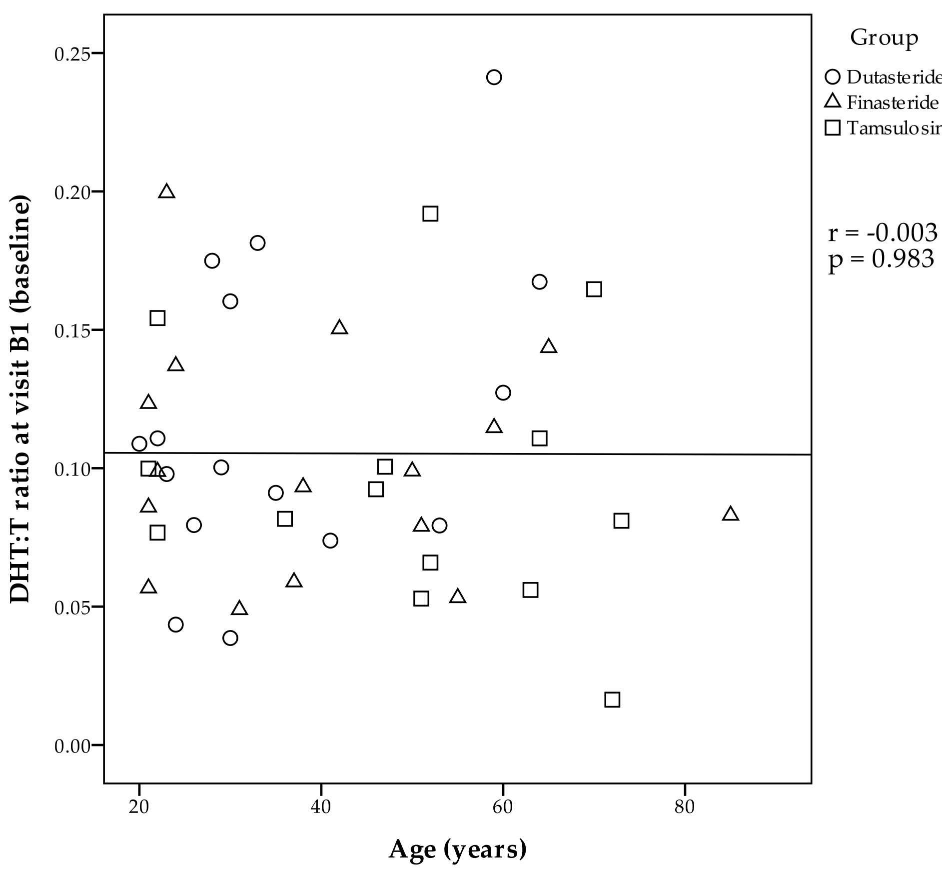

Figure 5.5 No correlation was observed between baseline serum DHT (dihydrotestosterone): T (testosterone) ratio with age, calculated with Pearson's correlation.

In addition to the study drug, several volunteers were receiving other prescribed medications prior to commencing participation in the study. These are summarised in Table 5-4. There were no alterations to medications or medication doses during the study period. Of note, the volunteer on thyroxine was clinically and biochemically euthyroid for several years, and thyroid function was normal on biochemical testing at the screening visit.

Indication	Class	Drug	Dutasteride group	Finasteride group	Tamsulosin group
Benign prostatic hyperplasia	α Blocker	Tamsulosin	A	D	F, G, H, I
	α Blocker	Indoramin		E	
	Anti-muscarinic	Tolterodine			F
	Anti-muscarinic	Solifenacin	A		
Dyslipidaemia	Lipid lowering drug	Simvastatin	B	E	J, K
Hypertension	Thiazide diuretic	Bendroflumethiazide	B		
	ARB	Losartan			J
Primary MI prevention	Anti-platelet	Aspirin	C		J
Dyspepsia	PPI	Lansoprazole			L
Hypothyroidism	Thyroid hormone	Thyroxine			I
Chronic back pain	μ Opiod receptor agonist	Tramadol (prn)			K
Mild COPD	β agonist inhaler (short-acting)	Salbutamol (prn)			K

Table 5-4 Medications at baseline, taken by participants in each group, with matching letters representing the same participant. ARB, angiotensin II receptor blocker; MI, myocardial infarction; PPI, proton pump inhibitor; prn, pro re nata (as needed); COPD, chronic obstructive pulmonary disease.

5.4.5 Pharmacodynamic responses to 5αR inhibitors

5.4.5.1 Circulating steroids

Serum androgen concentrations before and after drug treatment are summarised in Table 5-5.

There was a decrease from baseline in serum DHT concentrations, to approximately the same degree with both dutasteride and finasteride, and in many volunteers DHT was still quantifiable post treatment. There were 5 volunteers in the dutasteride group and 3 volunteers in the finasteride group in whom DHT was not detectable post treatment. As expected, DHT concentrations did not change with tamsulosin treatment. There was no significant change in testosterone with both 5αR inhibitors.

		Dutasteride	Finasteride	Tamsulosin	**Δ Dutasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Finasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Dutasteride vs. Δ Finasteride** Mean (95% CI)
	N	*16*	*16*	*14*			
T nmol/L	Pre ℞	25.4 (8.91)	20.7 (8.81)	21.2 (8.48)	Difference between groups: p=0.22		
	Post ℞	30.4 (12.80)	23.9 (7.93)	22.9 (9.48)			
	Change	*+ 5.1*	*+ 3.7*	*+ 1.7*			
DHT nmol/L	Pre ℞	2.9 (1.56)	2.1 (1.20)	2.0 (1.27)	***Difference between groups: p=0.02***		
	Post ℞	1.8 (1.65)	1.0 (0.64)	1.7 (1.07)	***-0.9 (-1.5; -0.2) p=0.01***	***-0.8 (-1.5; -0.2) p=0.01***	-0.0 (-0.6; 0.6) p=0.99
	Change	*- 1.2*	*-1.1*	*-0.3*			

Table 5-5 Serum testosterone (T) concentrations were unchanged, however serum dihydrotestosterone (DHT) was significantly decreased following three months of dutasteride or finasteride. Data are mean (SD). Change from baseline compared between groups by one-way ANOVA, followed by LSD post-hoc tests where significant. N, number; ℞, treatment.

5.4.5.2 Urinary steroids

Changes in urinary steroids, pertinent to pharmacodynamic responses to the drugs, are shown in Table 5-6 and Table 5-7. Other urinary steroid results, relating to HPA axis responses, are shown and discussed further in Chapter 6. Urinary 5α-reduced metabolites of both cortisol and androstenedione were significantly decreased following treatment with both dutasteride and finasteride. The decrease in αTHF with dutasteride and finasteride was also reflected in associated changes in the βTHF/αTHF and F/αTHF ratios. Androgen metabolites changed with both 5αR inhibitors. Compared to baseline, there was an expected decrease in androsterone (α) and there was a significant increase in the aetiocholanolone (β)/androsterone (α) ratio. There were not marked differences in extent of change in 5α-reduced metabolites between the dutasteride and finasteride groups, except in the increase in the aetiocholanolone/ androsterone ratio which was greater with dutasteride treatment.

		Dutasteride	Finasteride	Tamsulosin	**Δ D vs. Δ T** Mean (95% CI)	**Δ F vs. Δ T** Mean (95% CI)	**Δ D vs. Δ F** Mean (95% CI)
	N	*16*	*16*	*14*			
Andro (α) µg/day	Pre ℞	1806 (699.2)	2373 (1737.1)	2116 (1297.1)	***Difference between groups: p<0.001***		
	Post ℞	121 (73.0)	397 (310.0)	2036 (1258.5)	***-1605 (-2456; -754) p<0.001***	***-1896 (-2747;-1045) p<0.001***	291 (-1113; 531) p=0.48
	Change	*-1684*	*-1975*	*-79*			
Aetio (β) µg/day	Pre ℞	817 (403.4)	805 (604.5)	861 (484.6)	***Difference between groups: p<0.001†***		
	Post ℞	2461 (1350.5)	1710 (1002.9)	1029 (658.8)	***p<0.001***	***p=0.01***	p=0.28
	Change	*+1643*	*+905*	*+168*			
5αTHF µg/day	Pre ℞	1664 (1006.9)	1858 (1424.3)	1786 (1123.3)	***Difference between groups: p<0.001***		
	Post ℞	32 (36.2)	51 (53.6)	1773 (1153.9)	***-1620 (-2422; -817) p<0.001***	***-1794 (-2597; -992) p<0.001***	174 (-601; 950) p=0.65
	Change	*-1633*	*-1807*	*-13*			
5βTHF µg/day	Pre ℞	1724 (511.5)	1670 (649.2)	1793 (508.3)	Difference between groups: p=0.96		
	Post ℞	1718 (521.3)	1683 (506.9)	1742 (615.9)			
	Change	*-6*	*+14*	*-52*			

Table 5-6 Urinary androgen and glucocorticoid metabolites. There was a significant decrease in 5αTHF and androsterone following 5αR inhibition, together with a significant increase in aetiocholanolone (Aetio). Data are mean (SD). Change from baseline compared between groups by one-way ANOVA, with LSD post-hoc tests where significant, or †Kruskal Wallis test with pairwise comparisons. D, dutasteride; F, finasteride; T, tamsulosin; N, number; ℞, treatment; Andro, androsterone; THF, tetrahydrocortisol.

			Dutasteride	Finasteride	Tamsulosin	Δ D vs. Δ T Mean (95% CI)	Δ F vs. Δ T Mean (95% CI)	Δ D vs. Δ F Mean (95% CI)
		N	*16*	*16*	*14*			
Glucocorticoids	**βTHF/ αTHF**	Pre ℞	1.39 (0.78)	1.21 (0.73)	1.30 (0.67)	***Difference between groups: $p<0.001^{\dagger}$***		
		Post ℞	96.20 (66.72)	55.69 (33.36)	1.37 (0.90)	***$p<0.001$***	***$p<0.001$***	***$p=0.65$***
		Change	*+94.81*	*+54.48*	*+0.07*			
	F/ αTHF	Pre ℞	0.10 (0.10)	0.10 (0.05)	0.12 (0.11)	***Difference between groups: $p<0.001^{\dagger}$***		
		Post ℞	6.12 (3.68)	4.22 (2.33)	0.13 (0.12)	***$p<0.001$***	***$p<0.001$***	***$p=0.88$***
		Change	*+6.01*	*+4.11*	*+0.01*			
	F/ βTHF	Pre ℞	0.08 (0.05)	0.09 (0.04)	0.09 (0.05)	Difference between groups: $p=0.67$		
		Post ℞	0.08 (0.04)	0.08 (0.04)	0.09 (0.04)			
		Change	*+0*	*-0.01*	*0*			
Androgens	**Aetio (β) / Andro (α)**	Pre ℞	0.45 (0.18)	0.36 (0.20)	0.43 (0.14)	***Difference between groups: $p<0.001^{\dagger}$***		
		Post ℞	21.82 (9.73)	8.26 (15.55)	0.85 (1.43)	***$p<0.001$***	***$p=0.01$***	***$p=0.01$***
		Change	*+21.37*	*+7.89*	*+0.43*			

Table 5-7 Indices of pathways of A-ring reduction of glucocorticoids and androgens by 5αR were decreased relative to 5βR following three months of either dutasteride or finasteride. Data are mean (SD). Change from baseline compared between groups by one-way ANOVA (F/βTHF), or †Kruskal Wallis test with pairwise comparisons. D, dutasteride; F, finasteride; T, tamsulosin; N, number; ℞, treatment; THF, tetrahydrocortisol F, cortisol; Aetio, aetiocholanolone; Andro; androsterone.

5.4.6 Effects of 5αR inhibition on insulin sensitivity

5.4.6.1 Insulin sensitivity in the fasted state

Under fasting conditions, glucose was unchanged by treatment; however there was a trend towards an increase in insulin, and a significant increase in C-peptide in the dutasteride group (Table 5-8). HbA1c was not different. Measures calculated from glucose and insulin are summarised in Table 5-9. Here it is seen that HOMA-IR increased significantly from baseline with dutasteride only, while there was no difference in the QUICKI measure.

		Dutasteride	Finasteride	Tamsulosin	**Δ D vs. Δ T** Mean (95% CI)	**Δ F vs. Δ T** Mean (95% CI)	**Δ D vs. Δ F** Mean (95% CI)
	N	*16*	*16*	*14*			
Glucose mmol/L	Pre ℞	4.96 (0.49)	4.98 (0.50)	5.14 (0.37)	Difference between groups: p=0.34		
	Post ℞	5.09 (0.53)	4.93 (0.38)	5.06 (0.38)			
	Change	*+ 0.13*	*- 0.05*	*- 0.08*			
HbA1c nmol/mol	Pre ℞	35.2 (4.18)	35.1 (2.64)	36.2 (3.07)	Difference between groups: p=0.62†		
	Post ℞	35.6 (4.00)	35.9 (2.29)	36.6 (3.32)			
	Change	*+ 0.4*	*+ 0.9*	*+ 0.4*			
Insulin pmol/L	Pre ℞	58.8 (23.80)	54.1 (17.85)	63.1 (33.01)	*Difference between groups: p=0.07*		
	Post ℞	68.8 (30.01)	57.7 (19.13)	59.3 (33.06)			
	Change	*+ 10.0*	*+0.6*	*-3.9*			
	N	*15*	*16*	*14*	***Difference between groups: p=0.04***		
C-peptide pmol/L	Pre ℞	539 (156.9)	539 (172.9)	613 (295.7)	***100 (14; 187)***	11 (-74; 97)	***89 (5; 173)***
	Post ℞	615 (171.2)	526 (165.6)	588 (269.5)	***p=0.02***	p=0.79	***p=0.04***
	Change	*+ 76*	*- 13*	*- 25*			

Table 5-8 Three months of treatment with dutasteride (D) significantly increased fasting C-peptide, with a trend towards an increase in fasting insulin. There was no difference in fasting glucose or haemoglobin A1c (HbA1c) between groups. Data are mean (SD), change from baseline compared between groups by one way ANOVA, with LSD post-hoc tests where significant, other than HbA1c which was analysed by †Kruskal Wallis test. F, finasteride, T, tamsulosin; N, number; ℞, treatment.

		Dutasteride	Finasteride	Tamsulosin	**Δ Dutasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Finasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Dutasteride vs. Δ Finasteride** Mean (95% CI)
	N	*16*	*16*	*14*			
HOMA-IR	Pre ℞	1.89 (0.79)	1.73 (0.63)	2.09 (1.17)	***Difference between groups: p=0.03***		
	Post ℞	2.28 (1.06)	1.83 (0.65)	1.95 (1.19)	***0.53 (0.13; 0.93) p=0.01***	0.24 (-0.16; 0.63) p=0.23	0.29 (-0.09; 0.68) p=0.13
	Change	*+ 0.39*	*+ 0.10*	*- 0.14*			
QUICKI	Pre ℞	0.36 (0.03)	0.36 (0.02)	0.35 (0.03)	Difference between groups: p=0.11		
	Post ℞	0.35 (0.04)	0.35 (0.02)	0.35 (0.03)			
	Change	*- 0.007*	*- 0.003*	*- 0.004*			

Table 5-9 HOMA-IR (Homeostatic model assessment of insulin resistance) increased significantly following three months of treatment with dutasteride. QUICKI (Quantitative insulin sensitivity check index) was unaltered. Data are mean (SD). Change from baseline compared between groups by one-way ANOVA, followed by LSD post-hoc tests where significant. N, number; ℞, treatment.

5.4.6.2 Glucose disposal, glucose production and lipolysis during a hyperinsulinaemic euglycaemic clamp

5.4.6.2.1 Glucose concentrations during clamp

The clamp glucose target of 4.5 – 5.5 mmol/L was successfully achieved, as shown in Figure 5.6.

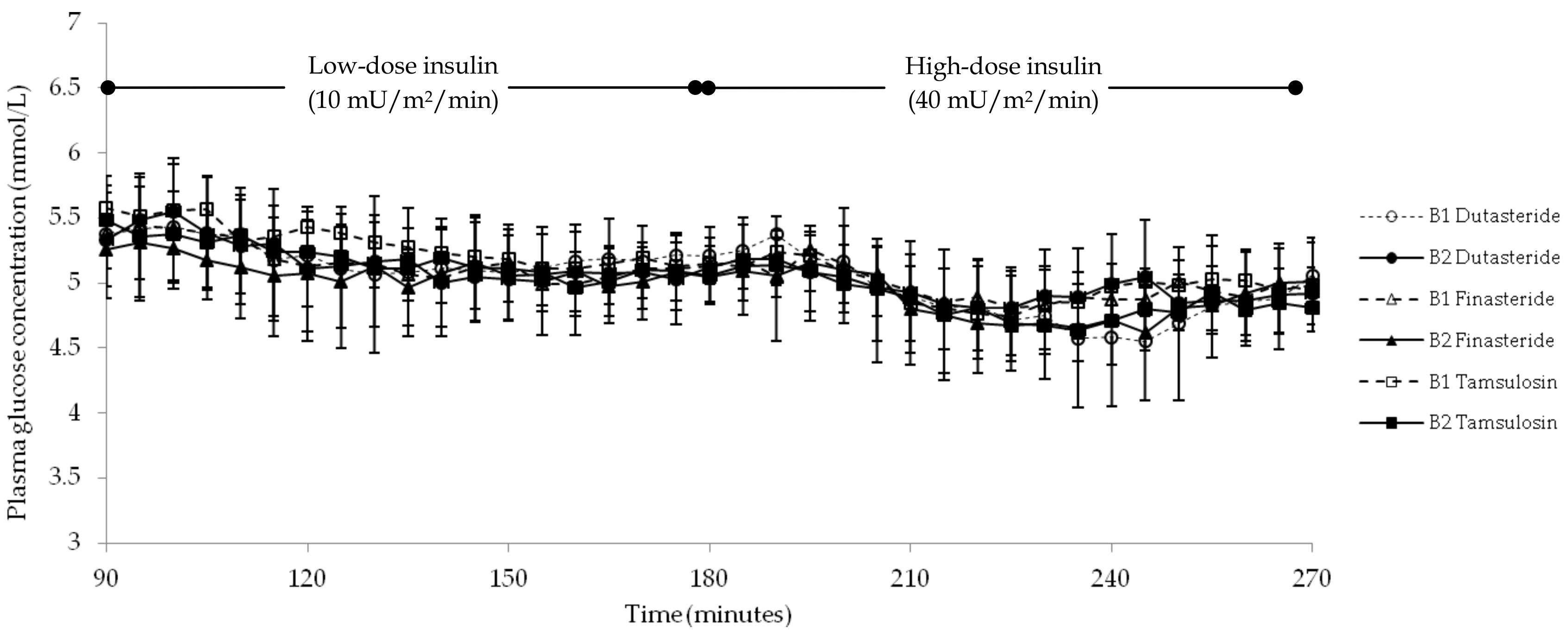

Figure 5.6 Plasma glucose concentrations (mmol/L) during hyperinsulinaemic phases of clamps conducted before (B1) or after (B2) three months of treatment with dutasteride, finasteride or tamsulosin; target was 4.5-5.5 mmol/L and this was successfully achieved. Data are mean, error bars indicate standard deviation.

5.4.6.2.2 Tracer: tracee ratios (TTRs) for d2-glucose: glucose

TTRs are shown in Figure 5.7. d2-Glucose: glucose ratios achieved steady state in each phase of the clamp. Relative standard deviations between samples in each phase ranged from 0.4 – 3.8%. TTR increased between baseline and the end of the tracer only infusion period. There was no difference in TTR at the end of low dose insulin. TTR decreased with high dose insulin as glucose uptake was stimulated and glucose infusion increased.

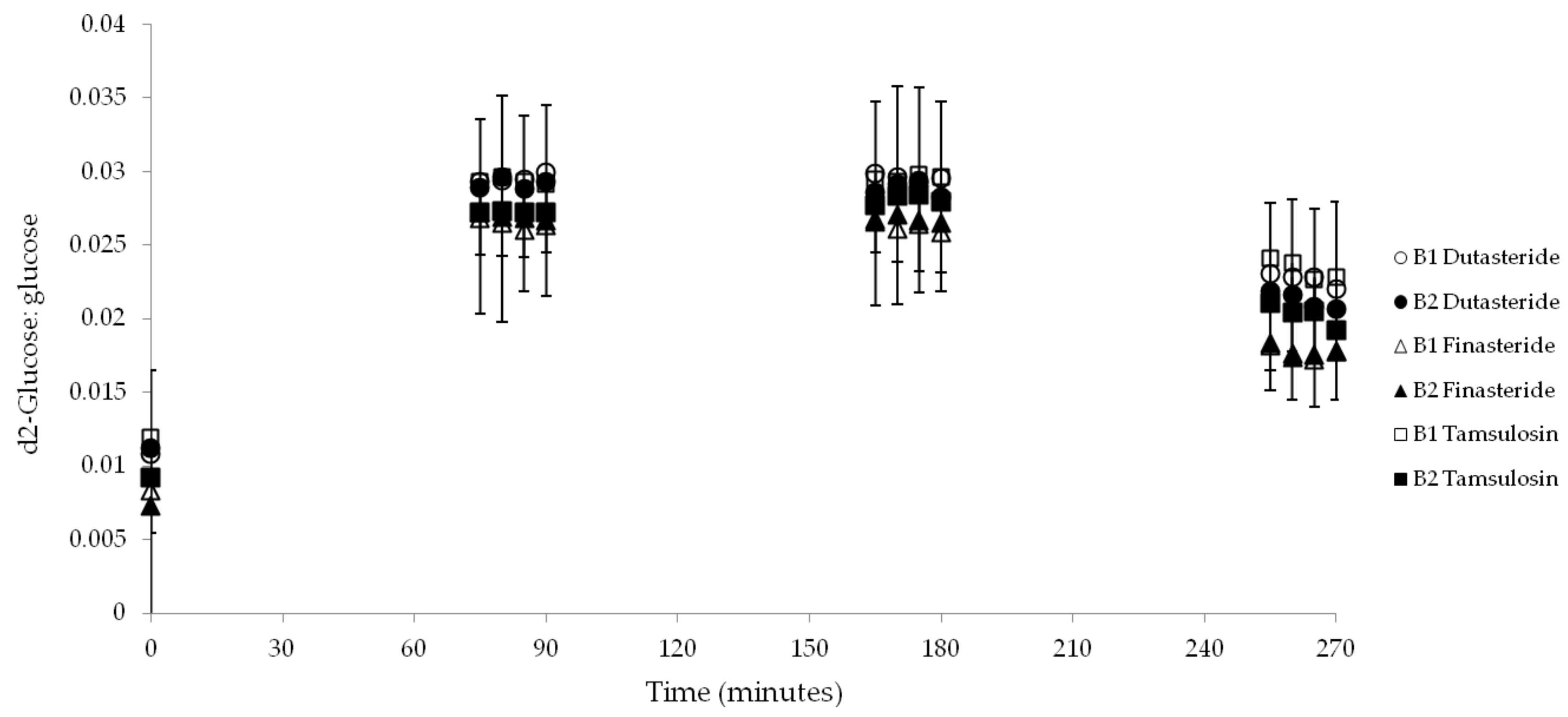

Figure 5.7 d2-Glucose: glucose tracer: tracee ratios during hyperinsulinaemic euglycaemic clamps, before (B1) and after (B2) three months of dutasteride, finasteride or tamsulosin. Data are mean, error bars indicate standard deviation.

5.4.6.2.3 Insulin concentrations during clamp

Insulin concentrations at baseline were presented in Section 5.4.6.1. Insulin concentrations during the clamp are summarised in Table 5-10.

During the first phase of the clamp when d2-glucose and d5-glycerol were administered, insulin concentrations were increased post-treatment in the dutasteride group only, compared to control.

Differences in insulin concentrations following both low-dose and high-dose insulin infusion were not different between treatment groups.

Plasma insulin		Dutasteride	Finasteride	Tamsulosin	**Δ Dutasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Finasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Dutasteride vs. Δ Finasteride** Mean (95% CI)
	N	*16*	*16*	*14*			
Tracer only (pre-insulin infusion)	Pre ℞	31.5 (13.11)	35.6 (12.39)	36.9 (19.74)	***Difference between groups: p=0.03***		
	Post ℞	37.1 (18.11)	34.6 (11.10)	30.8 (11.18)	***11.7 (3.2; 20.3) p=0.008***	5.1 (-3.5; 13.6) p=0.24	6.6 (-1.6; 14.9) p=0.11
	Change	*+ 5.6*	*- 1.0*	*- 6.1*			
Low dose insulin infusion	Pre ℞	85.8 (35.00)	83.8 (23.24)	82.1 (25.89)	Difference between groups: p=0.84		
	Post ℞	91.1 (39.49)	83.1 (23.25)	81.5 (22.71)			
	Change	*+ 5.4*	*- 0.8*	*- 0.6*			
	N	*15*	*16*	*14*			
High dose insulin infusion	Pre ℞	306.7 (72.73)	295.3 (53.34)	258.7 (57.55)	Difference between groups: p=0.51		
	Post ℞	326.2 (56.08)	303.3 (76.24)	291.8 (56.59)			
	Change	*+ 20.3*	*+ 8.0*	*+ 33.0*			

Table 5-10 Plasma insulin concentrations (pmol/L) following tracer (d2-glucose and d5-glycerol) only phase of clamp increased following 3 months of treatment with dutasteride. Insulin concentrations following low dose (10 mU/m^2/min) or high dose (40 mU/m^2/min) phases of the clamp did not differ between groups. Data are mean (SD), and comparisons by one-way ANOVA of change from baseline, with LSD post-hoc testing where significant. N, number; ℞, treatment.

5.4.6.2.4 Endogenous glucose production

There were no differences in fasting plasma glucose concentrations, and these data were presented in Section 5.4.6.1. Endogenous glucose production (EGP) at baseline and with low-dose insulin is summarised in Table 5-11, demonstrating no differences between treatment groups.

EGP		Dutasteride	Finasteride	Tamsulosin	**Difference**
	N	*16*	*16*	*14*	
Post-tracer µmol/kg/min	Pre ℞	7.24 (1.50)	7.91 (1.11)	7.38 (1.66)	p=0.10
	Post ℞	7.13 (1.26)	7.71 (0.97)	7.74 (1.34)	
	Change	*-0.11*	*-0.20*	*+0.36*	
Low-dose insulin µmol/kg/min	Pre ℞	3.95 (3.32)	4.47 (2.22)	5.02 (1.90)	p=0.77
	Post ℞	4.24 (2.16)	4.23 (2.47)	4.93 (2.25)	
	Change	*+0.29*	*-0.24*	*-0.09*	
Post-tracer µmol/kgFFM /min	Pre ℞	9.03 (2.06)	10.23 (1.78)	9.83 (2.17)	p=0.24
	Post ℞	9.10 (2.20)	10.02 (1.82)	10.21 (1.76)	
	Change	*+0.07*	*-0.21*	*+0.38*	
Low-dose insulin µmol/kgFFM /min	Pre ℞	5.10 (3.95)	5.80 (3.10)	6.67 (2.39)	p=0.72
	Post ℞	5.54 (3.08)	5.54 (3.44)	6.56 (2.96)	
	Change	*+0.44*	*-0.25*	*-0.11*	

Table 5-11 Endogenous glucose production (EGP) during first two phases of hyperinsulinaemic euglycaemic clamp did not change with three months of dutasteride, finasteride or tamsulosin. EGP presented were calculated following 90 minutes of tracer infusion (d2-glucose and d5-glycerol), and following low-dose (10 mU/m^2/min) insulin infusion; effect of tracers was evident with $p<0.001$ for change in d2-glucose:glucose ratio from baseline to low-dose insulin, and effect of insulin was evident with $p<0.001$ for change in insulin concentration from pre-insulin to low-dose insulin. Data are mean (SD). Change from baseline compared between groups by one-way ANOVA. N, number; ℞, treatment; FFM, fat-free mass.

High dose insulin was administered to assess peripheral glucose uptake. There may be, however, some remaining hepatic glucose output during this phase. Although d2-glucose could be detected in all subjects, in 36/92 clamps apparent negative values were obtained for EGP at high dose insulin, confirming suppression of hepatic glucose output particularly in very insulin sensitive volunteers. This did however mean calculations to quantify EGP during high dose insulin were unreliable, and only peripheral glucose uptake was formally measured and these results are shown below in Sections 5.4.6.2.5 and 5.4.6.2.6.

5.4.6.2.5 Glucose infusion rates during high dose insulin (M value)

There was a decrease from baseline in the M value in the dutasteride group compared to both the finasteride and tamsulosin groups. There was no difference between the finasteride and tamsulosin groups.

M values are shown in Table 5-12, demonstrating that the inferred decrease in insulin sensitivity with dutasteride compared to the other two groups persists when calculated by weight or by fat-free mass, which is important given the increase in fat mass seen in this group (Section 5.4.8.2). Individual changes in M value are shown in Figure 5.8. It can be seen that overall those

on dutasteride require less glucose to maintain euglycaemia, whereas those on finasteride and tamsulosin require more.

Subtle variations in glucose concentrations achieved during clamps are inevitable, as shown in Section 5.4.6.2.1. The values included in the final analysis are those without adjustment for glucose concentration, however when data were adjusted to allow for slight variations in glucose concentrations during clamp (by using the ratio of glucose infusion rate/ glucose concentration, rather than the infusion rate alone), changes demonstrated remained (data not shown).

M value		Dutasteride	Finasteride	Tamsulosin	**Δ Dutasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Finasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Dutasteride vs. Δ Finasteride** Mean (95% CI)
	N	*15*	*16*	*14*			
mL/hr	Pre ℞	151.6 (50.5)	142.1 (62.8)	97.6 (43.2)	***Difference between groups: p=0.003***		
	Post ℞	130.6 (60.0)	167.9 (80.4)	123.3 (52.0)	***-46.7 (-77.1; -16.3) p=0.003***	+ 0.1 (-29.8; 30.1) p=0.99	***-46.8 (-76.2;-17.4) p=0.003***
	Change	*- 21.00*	*+ 25.83*	*+ 25.71*			
µmol/kg/ min	Pre ℞	37.7 (14.8)	31.6 (14.0)	23.3 (12.8)	***Difference between groups: p=0.003***		
	Post ℞	32.4 (17.1)	37.5 (16.6)	29.7 (15.8)	***-11.6 (-18.9; -4.3) p=0.003***	-0.5 (-7.7; 6.6) p=0.88	***-11.1 (-18.2; -4.0) p=0.003***
	Change	*- 5.27*	*+ 5.83*	*+ 6.36*			
µmol/ kgFFM/min	Pre ℞	45.2 (15.3)	40.0 (16.3)	30.7 (15.7)	***Difference between groups: p=0.002***		
	Post ℞	39.0 (18.5)	47.8 (20.5)	38.3 (17.7)	***-13.8 (-22.6; -4.9) p=0.002***	0.2 (-8.5; 8.9) p=0.96	***-14.0 (-22.5; -5.5) p=0.002***
	Change	*- 6.17*	*+ 7.82*	*+ 7.59*			

Table 5-12 M value (mean steady state glucose infusion rate) during high-dose (40 mU/m^2/min) insulin infusion phase of the clamp, significantly decreased after 3 months of treatment with dutasteride, compared to both finasteride and tamsulosin. M value shown in mL/hr (infusion as given), as µmol/kg/min (adjusted for body weight), and as µmol/kgFFM/min (adjusted for fat mass). Data are mean (SD) and change from baseline is compared between groups by one-way ANOVA followed by LSD post-hoc tests. N, number; ℞, treatment; FFM, fat-free mass.

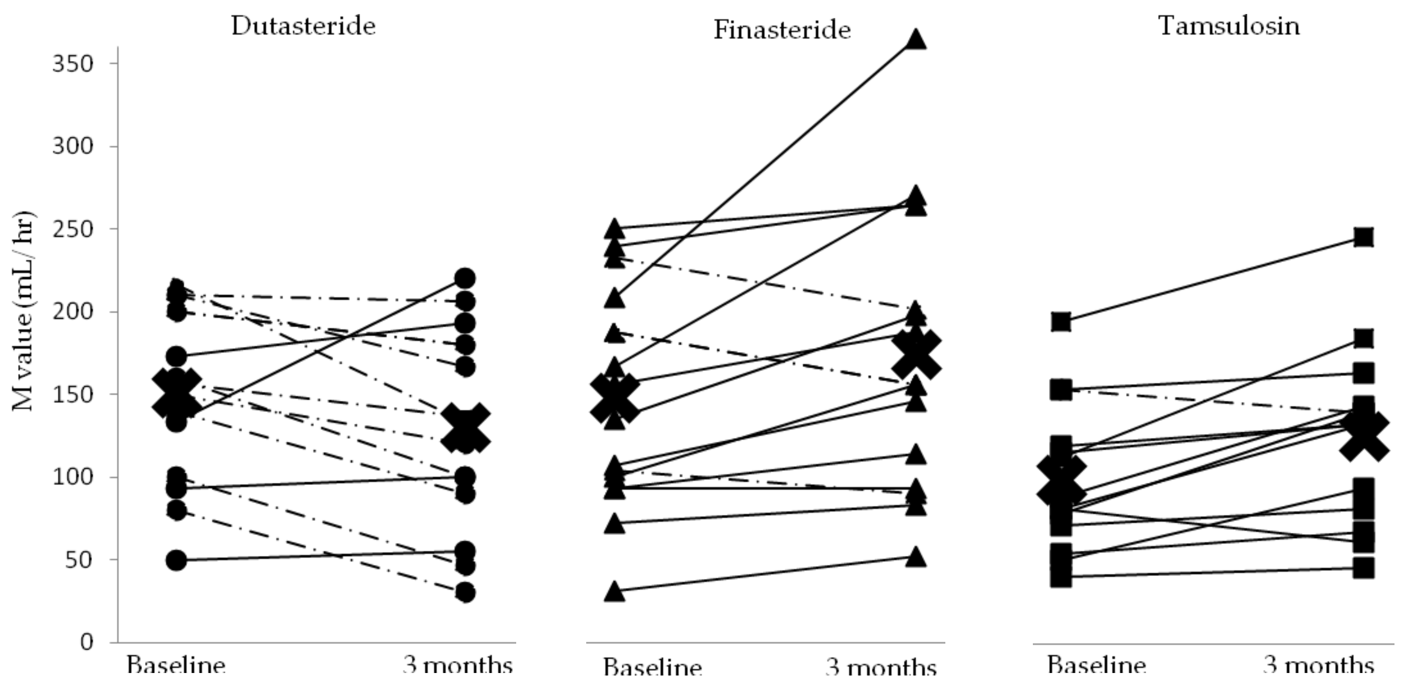

Figure 5.8 M values (mean steady state glucose infusion rate) during high dose insulin infusion (40 mU/ m^2/ minute), with value calculated as average glucose infusion rate between timepoints +255 to +270 minutes. Results presented from before and after three months of dutasteride, finasteride or tamsulosin. Increased (or unchanged) and decreased glucose infusion rates required at follow-up clamp indicated by solid and interrupted lines respectively. Crosses indicate average value for each group.

5.4.6.2.6 Glucose rate of disposal (Rd glucose) during high dose insulin

Rd glucose can be derived from the M value and TTR, allowing for the rate of glucose infusion and any residual hepatic glucose output remaining. As shown in Table 5-13, results were essentially identical to the M value, with a decrease from baseline in Rd glucose following dutasteride treatment.

Rd glucose		Dutasteride	Finasteride	Tamsulosin	**Δ Dutasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Finasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Dutasteride vs. Δ Finasteride** Mean (95% CI)
	N	*15*	*16*	*14*			
μmol/ kg/min	Pre ℞	34.8 (13.3)	29.3 (13.0)	21.6 (11.9)	***Difference between groups: p=0.003***		
	Post ℞	30.0 (4.1)	34.7 (3.8)	27.5 (14.6)	***-10.7 (-17.5; -4.0) p=0.003***	-0.5 (-7.2; 6.2) p=0.88	***-10.2 (-16.8; -3.7) p=0.003***
	Change	*- 4.9*	*+ 5.4*	*+ 5.9*			
μmol/ kgFFM/min	Pre ℞	41.9 (13.7)	37.0 (15.1)	28.4 (14.5)	***Difference between groups: p=0.002***		
	Post ℞	36.1 (17.1)	44.2 (19.0)	35.4 (16.4)	***-12.7 (-20.9; -4.5) p=0.003***	0.2 (-7.8; 8.3) p=0.95	***-12.9 (-20.8; -5.0) p=0.002***
	Change	*- 5.7*	*+ 7.2*	*+ 7.0*			

Table 5-13 Rate of disposal (Rd) of glucose during high dose insulin decreased significantly following three months of dutasteride, compared to both finasteride and tamsulosin. Results were the same when expressed per kg fat-free mass (FFM). Effect of tracers was evident with p<0.001 for decrease in d2-glucose:glucose ratio from low to high dose insulin, and effect of insulin was evident with p<0.001 for change in insulin concentration from low to high dose insulin. Data are mean (SD), and change from baseline compared between groups by one-way ANOVA with LSD post-hoc tests. N, number; ℞, treatment.

5.4.6.2.7 Tracer: Tracee Ratios (TTRs) for d5-glycerol: glycerol

d5-Glycerol: glycerol TTRs are demonstrated in Figure 5.9. In comparison to glucose tracer kinetics there was increased variability in d5-glycerol: glycerol TTRs, largely due to difficulty in accurately quantifying d5-glycerol at low concentrations. Despite these analytical issues however, relative standard deviation at each steady state phase ranged from 1.2 – 11.7%, and the majority were below 5%.

TTRs increased from zero at baseline, following commencement of tracer infusion. There was an increase in TTR between baseline and the end of low dose insulin, and further with high dose insulin during the final phase of the clamp.

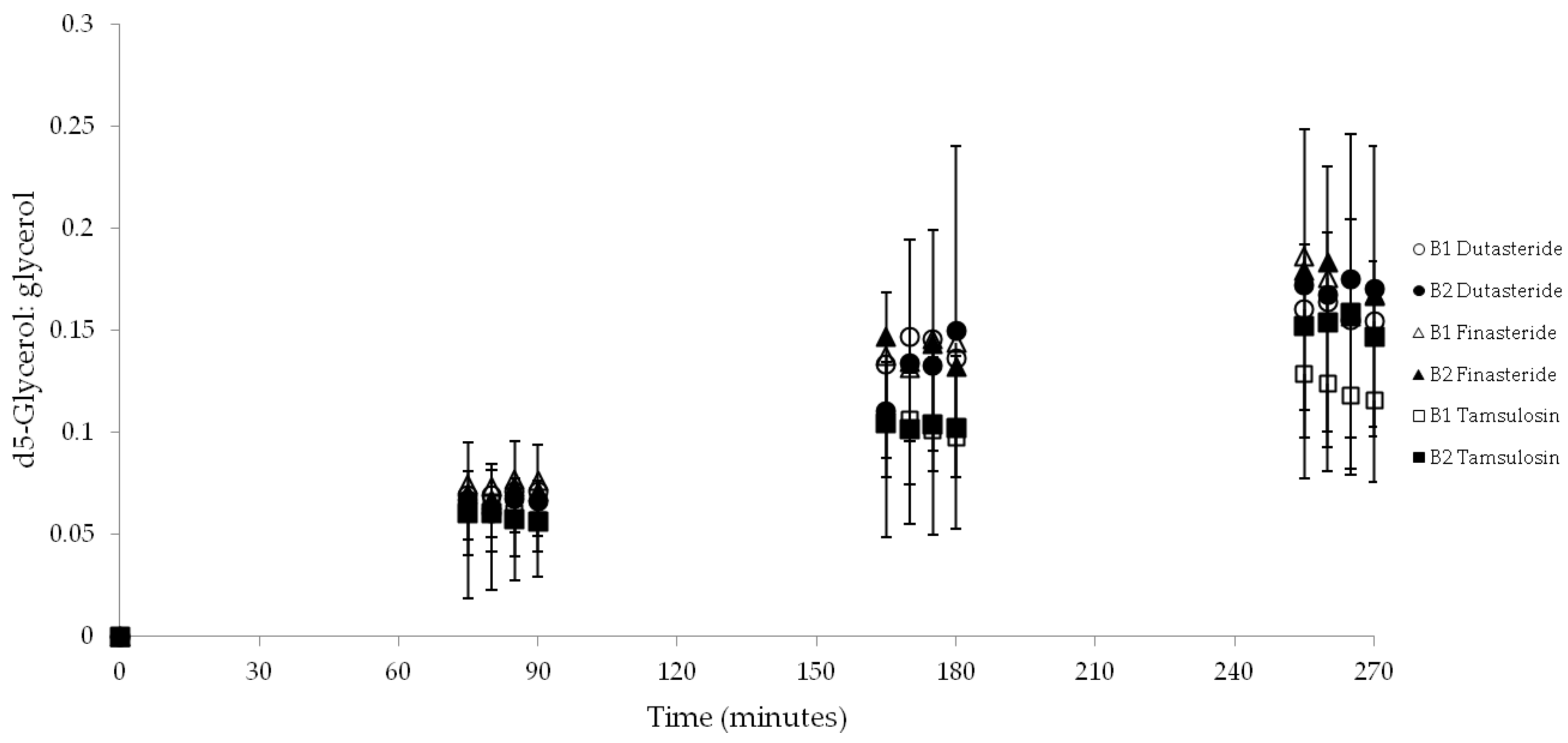

Figure 5.9 d5-Glycerol: glycerol tracer: tracee ratios during hyperinsulinaemic euglycaemic clamp, before (B1) and after (B2) three months of dutasteride, finasteride or tamsulosin. Data are mean, error bars indicate standard deviation.

5.4.6.2.8 Glycerol concentrations during clamp

Glycerol concentrations were suppressed in response to low-dose insulin and further in response to high dose insulin, reflecting inhibition of lipolysis by insulin, as shown in Figure 5.10. However the changes in glycerol concentration following treatment were not different between groups, as shown in Table 5-14.

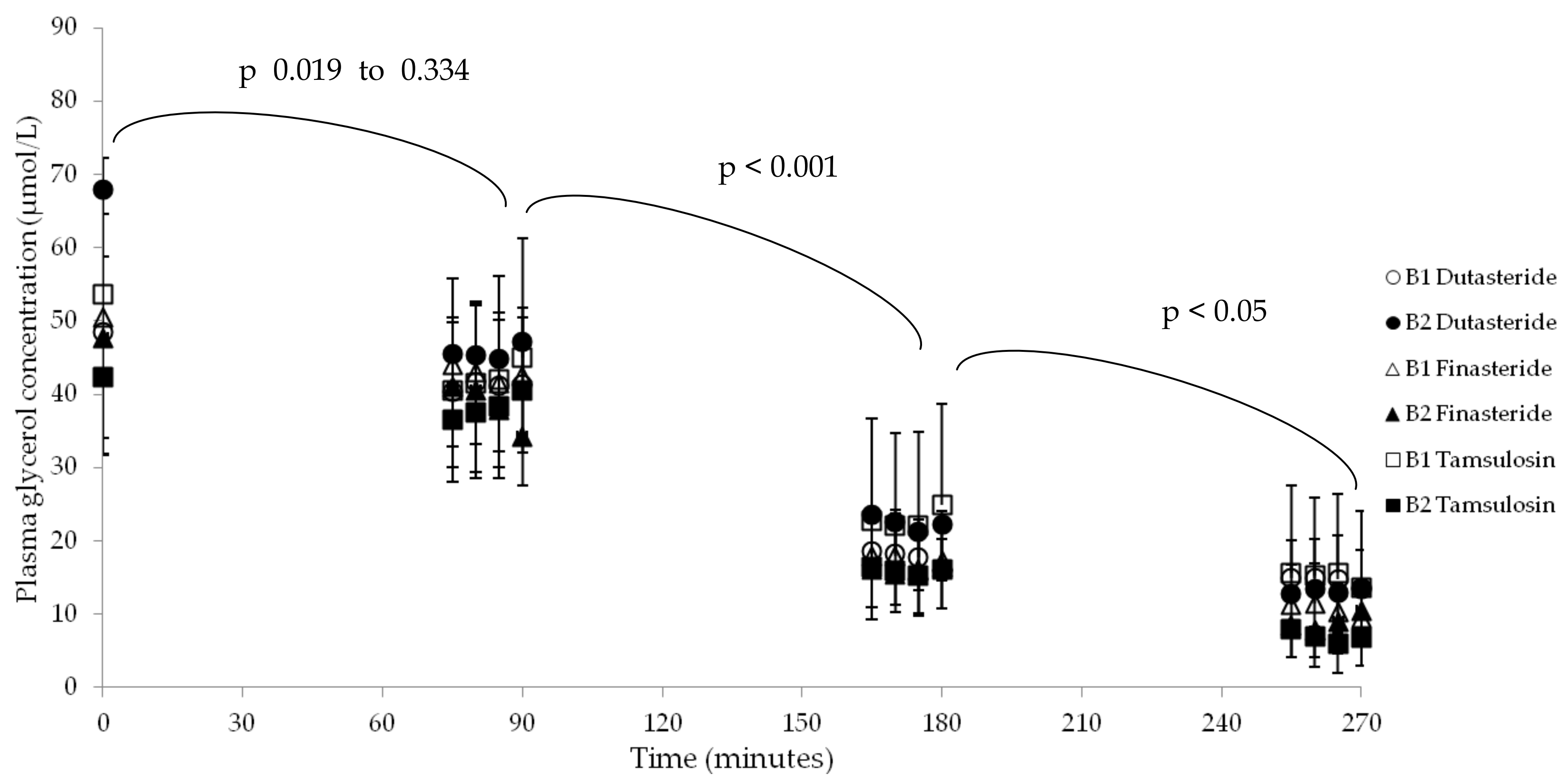

Figure 5.10 Plasma glycerol concentrations were suppressed during hyperinsulinaemic euglycaemic clamps. Bars and p values indicate difference between phases of the clamp, calculated for each group by paired t-tests comparing mean values for each phase. Data are mean; error bars indicate 95% confidence interval. B1, before treatment; B2, after treatment.

Plasma glycerol		Dutasteride	Finasteride	Tamsulosin	**Difference between groups**
	N	*16*	*16*	*14*	
Fasting	Pre ℞	48.4 (21.11)	50.4 (28.98)	53.6 (35.55)	p=0.10
	Post ℞	67.9 (73.44)	47.7 (27.94)	42.3 (20.1)	
	Change	*+19.5*	*-2.7*	*-11.3*	
Post-tracer	Pre ℞	41.2 (20.12)	42.8 (18.95)	42.3 (23.66)	p=0.28
	Post ℞	45.7 (25.27)	38.4 (18.72)	38.2 (16.00)	
	Change	*+4.5*	*-4.4*	*-4.0*	
Low dose insulin	Pre ℞	18.0 (10.48)	17.4 (12.27)	22.6 (24.93)	p=0.17
	Post ℞	22.4 (15.85)	16.0 (11.95)	16.0 (9.64)	
	Change	*+4.4*	*-1.5*	*-6.7*	
	N	*15*	*16*	*14*	
High dose insulin	Pre ℞	14.7 (10.68)	10.6 (9.57)	15.0 (20.81)	p=0.21
	Post ℞	13.1 (11.13)	8.9 (8.54)	6.9 (7.40)	
	Change	*-1.8*	*-1.7*	*-8.1*	

Table 5-14 Plasma glycerol concentrations (µmol/L) during hyperinsulinaemic euglycaemic clamps did not differ following three months of either dutasteride, finasteride or tamsulosin. Glycerol concentrations are from fasting samples, following tracer infusion only (average of +75 to +90 minutes), following low dose insulin (average of +165 to +180 minutes) and following high dose insulin (average of +255 to +270 minutes). Data are mean (SD) and change from baseline compared between groups by one-way ANOVA. N, number; ℞, treatment.

5.4.6.2.9 Glycerol kinetics

There were no differences between treatment groups in the rate of appearance (Ra) of glycerol, either at baseline following infusion of tracer only, or following low dose insulin infusion intended for suppression of lipolysis, although there was considerable heterogeneity in response to treatment.

Ra glycerol		Dutasteride	Finasteride	Tamsulosin	**Difference between groups**
	N	*16*	*16*	*14*	
Post-tracer µmol/kg/min	Pre ℞	2.02 (1.04)	1.80 (0.87)	2.35 (1.12)	p=0.78
	Post ℞	2.14 (1.08)	1.76 (0.44)	2.29 (1.07)	
	Change	*+ 0.12*	*- 0.04*	*- 0.05*	
Low-dose insulin µmol/kg/min	Pre ℞	1.00 (0.56)	1.02 (0.54)	1.49 (1.03)	p=0.27
	Post ℞	1.07 (0.47)	1.00 (0.51)	1.21 (0.31)	
	Change	*+ 0.08*	*- 0.02*	*- 0.28*	
Post-tracer µmol/kgFFM/min	Pre ℞	2.54 (1.35)	2.32 (1.11)	3.14 (1.46)	p=0.60
	Post ℞	2.78 (1.66)	2.27 (0.64)	3.04 (1.47)	
	Change	*+ 0.24*	*- 0.04*	*- 0.09*	
Low-dose insulin µmol/kgFFM/min	Pre ℞	1.23 (0.60)	1.32 (0.72)	1.99 (1.33)	p=0.16
	Post ℞	1.39 (0.69)	1.30 (0.67)	1.60 (0.41)	
	Change	*+ 0.16*	*- 0.02*	*- 0.39*	

Table 5-15 Rate of appearance (Ra) of glycerol during hyperinsulinaemic euglycaemic clamps did not change after three months of dutasteride, finasteride or tamsulosin. Ra glycerol calculated following 90 minutes of tracer infusion (d2-glucose and d5-glycerol), and following low-dose (10 mU/m^2/min) insulin infusion. Effect of tracers was evident with $p<0.001$ for change in d5-glycerol:glycerol ratio from baseline to low-dose insulin, and effect of insulin was evident with $p<0.001$ for change in insulin concentration from pre-insulin to low-dose insulin. Results presented both per kg and per kg fat-free mass (FFM). Data are mean (SD); change from baseline is compared between groups by one-way ANOVA. N, number; ℞, treatment.

5.4.6.2.10 Non-esterified fatty acids (NEFAs)

NEFAs were suppressed with low-dose insulin infusion in all groups, with results shown in Figure 5.11. However, there was less suppression of NEFAs with low-dose insulin infusion following three months of dutasteride treatment. Changes in fasting NEFAs and at the end of the tracer only infusion period were not significantly different. Results are summarised in Table 5-16.

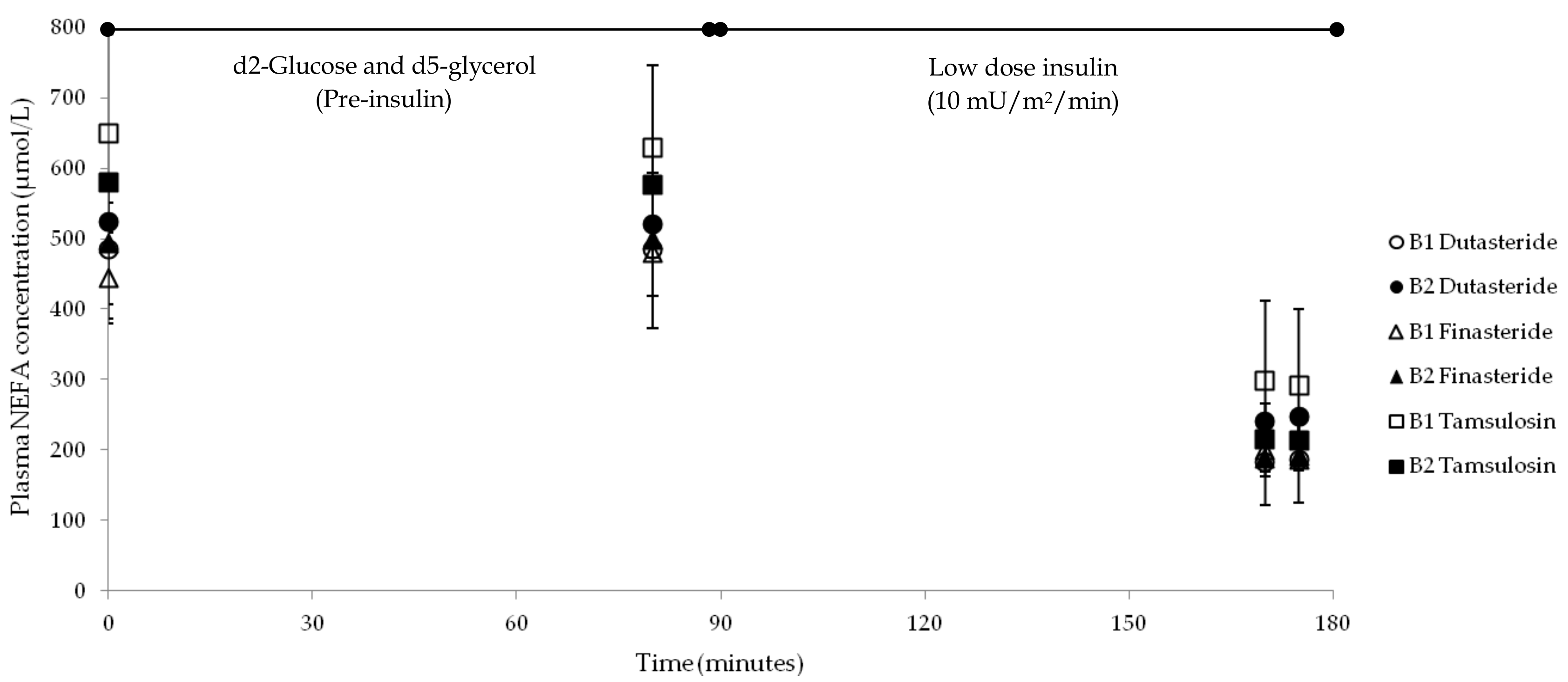

Figure 5.11 Plasma non-esterified fatty acid (NEFA) concentrations at baseline, following infusion of deuterated (d2-glucose and d5-glycerol) tracers, and following low dose (10 mU/m^2/min) insulin infusion. NEFA concentrations suppressed with insulin infusion compared to pre-insulin concentrations ($p<0.001$ by paired t-tests).

NEFAs		Dutasteride	Finasteride	Tamsulosin	Δ **Dutasteride vs. Δ Tamsulosin** Mean (95% CI)	Δ **Finasteride vs. Δ Tamsulosin** Mean (95% CI)	Δ **Dutasteride vs. Δ Finasteride** Mean (95% CI)
	N	*16*	*16*	*14*			
Fasting μM	Pre ℞	485.1 (212.59)	443.9 (219.10)	649.4 (267.30)	Difference between groups: p=0.37		
	Post ℞	523.8 (278.72)	493.1 (175.35)	580.2 (382.73)			
	Change	*+38.7*	*+49.2*	*-69.2*			
Post-tracer μM	Pre ℞	484.2 (209.82)	479.6 (233.41)	628.9 (223.76)	Difference between groups: p=0.46		
	Post ℞	520.1 (206.62)	497.5 (253.38)	577.2 (199.31)			
	Change	*+35.9*	*+17.9*	*-51.7*			
Low dose insulin μM	Pre ℞	184.9 (111.62)	193.4 (131.70)	295.1 (210.35)	***Difference between groups: p=0.041***		
	Post ℞	245.2 (146.37)	189.7(134.22)	214.1 (82.83)	***141.4 (32.7; 250.1) p=0.01***	77.3 (-31.4; 186.0) p=0.16	64.0 (-41.0; 169.0) p=0.23
	Change	*+60.3*	*-3.7*	*-81.0*			

Table 5-16 Plasma non-esterified fatty acids (NEFAs) were significantly less suppressed with low-dose insulin infusion following 3 months of dutasteride versus tamsulosin. Concentrations were measured prior to the clamp ("fasting"), at the end of the tracer (d2-glucose and d5-glycerol, "post-tracer") infusion, and at the end of 90 minutes of low-dose insulin infusion. Data are mean (SD) and change from baseline compared between groups by one-way ANOVA with LSD posthoc tests where significant. N, number; ℞, treatment.

5.4.7 Effect of 5αR inhibition on blood pressure and heart rate

Following three months of treatment, there were no differences in blood pressure or heart rate, as shown in Table 5-17. Of note, the control group tamsulosin (an α blocker) did not have any significant effect on these parameters.

		Dutasteride	Finasteride	Tamsulosin	**Difference between groups**
	N	*16*	*16*	*14*	
SBP mmHg	Pre ℞	131.0 (11.43)	136.4 (14.91)	138.8 (18.26)	p=0.56
	Post ℞	134.6 (15.98)	140.4 (9.77)	137.6 (15.22)	
	Change	*+ 3.6*	*+ 4*	*- 1.2*	
DBP mmHg	Pre ℞	77.6 (9.96)	78.4 (11.19)	81.3 (8.85)	p=0.60
	Post ℞	81.5 (10.03)	80.4 (6.24)	82.2 (9.17)	
	Change	*+ 3.9*	+ 2	*+ 0.9*	
HR bpm	Pre ℞	72 (11.3)	65 (9.4)	74 (16.5)	p=0.70
	Post ℞	70 (11.1)	66 (6.1)	73 (14.9)	
	Change	- 2	*+ 0.9*	*- 0.3*	

Table 5-17 Three months of either dutasteride, finasteride or tamsulosin did not change systolic blood pressure (SBP), diastolic blood pressure (DBP) or heart rate (HR). Data are mean (SD), and change from baseline is compared between groups by one way ANOVA. N, number; ℞, treatment; bpm, beats per minute.

5.4.8 Effect of 5αR inhibition on anthropometric measurements and body composition

5.4.8.1 Weight, BMI and WHR

There were no significant differences in body weight, BMI or WHR, as shown in Table 5-18.

		Dutasteride	Finasteride	Tamsulosin	**Difference between groups**
	N	*16*	*16*	*14*	
Weight kg	Pre ℞	77.4 (13.18)	83.8 (14.10)	80.5 (10.41)	p=0.17
	Post ℞	78.3 (12.32)	83.2 (13.49)	80.5 (11.03)	
	Change	*+ 0.9*	*- 0.6*	*0*	
BMI kg/m²	Pre ℞	25.3 (4.41)	26.8 (3.82)	25.5 (2.76)	p=0.14
	Post ℞	25.6 (4.30)	26.6 (3.47)	25.6 (3.19)	
	Change	*+ 0.3*	*- 0.2*	*+ 0.1*	
WHR	Pre ℞	0.90 (0.08)	0.89 (0.07)	0.93 (0.06)	p=0.96
	Post ℞	0.90 (0.06)	0.90 (0.06)	0.93 (0.05)	
	Change	*0*	*+ 0.1*	*0*	

Table 5-18 There was no change in weight, body mass index (BMI) or waist: hip ratio (WHR) following three months of dutasteride, finasteride or tamsulosin. Data are mean (SD), and change from baseline is compared between groups by one way ANOVA. N, number; ℞, treatment.

5.4.8.2 Body fat

Results demonstrate an increase in body fat with dutasteride treatment, compared to tamsulosin, and a trend towards an increase when compared to finasteride. These changes were seen with both absolute (kg) measurements, as well as proportional (%) measurements. Data are shown in Table 5-19.

		Dutasteride	Finasteride	Tamsulosin	**Δ Dutasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Finasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Dutasteride vs. Δ Finasteride** Mean (95% CI)
	N	*16*	*16*	*14*			
Body fat kg	Pre ℞	16.5 (8.70)	18.9 (6.97)	20.1 (5.94)	***Difference between groups: p=0.048***		
	Post ℞	17.8 (8.52)	18.7 (6.39)	19.6 (6.82)	***1.7 (0.3; 3.2) p=0.02***	0.4 (-1.1; 1.8) p=0.63	*1.4 (-0.04; 2.8) p=0.06*
	Change	*+ 1.3*	*- 0.2*	*- 0.5*			
Body fat %	Pre ℞	19.8 (8.49)	22.1 (6.62)	24.7 (5.99)	***Difference between groups: p=0.02***		
	Post ℞	21.5 (8.36)	22.3 (6.79)	24.0 (6.87)	***2.4 (0.7; 4.0) p=0.006***	0.9 (-0.7; 2.6) p=0.27	*1.4 (-0.2; 3.0) p=0.08*
	Change	*+ 1.7*	*+ 0.2*	*- 0.8*			

Table 5-19 Body fat (assessed by electrical bioimpedance) increased following three months of treatment with dutasteride. Data are mean (SD). Change from baseline compared between groups by LSD post-hoc tests following a statistically significant one-way ANOVA. N, number; ℞, treatment.

5.4.8.3 Fat distribution

5.4.8.3.1 Abdominal visceral and subcutaneous adipose quantified by MRI

Results from the 40 structural scans performed are summarised in Table 5-20. The increase in body fat with dutasteride treatment (Section 5.4.8.2) was not accompanied by an increase in either visceral or subcutaneous abdominal adipose on MRI. There was also no change in the ratio of visceral to subcutaneous fat. In the absence of a baseline measure, relationships between abdominal fat and other potentially relevant covariates (including weight, BMI, body fat, WHR) were explored, with no significant differences in any measure.

	Dutasteride	Finasteride	Tamsulosin	**Difference between groups**
N	*13*	*16*	*11*	
VF (kg)	0.09 (0.04)	0.07 (0.04)	0.10 (0.05)	p=0.16
SCF (kg)	0.22 (0.10)	0.21 (0.10)	0.20 (0.08)	p=0.85
AV (kg)	0.56 (0.15)	0.55 (0.12)	0.55 (0.12)	p=0.98
VF (%)	15.9 (4.4)	12.3 (5.8)	17.0 (7.0)	p=0.09
SCF (%)	37.8 (9.9)	35.9 (11.4)	35.4 (7.9)	p=0.81
VF:SCF	0.45 (0.18)	0.39 (0.22)	0.50 (0.21)	p=0.40

Table 5-20 The amounts of visceral fat (VF), subcutaneous fat (SCF) and abdominal volume (AV) measured by magnetic resonance imaging at L4/5 level after three months of treatment with dutasteride, finasteride or tamsulosin were not different between groups. The ratio of VF:SCF also did not differ between groups. Data are mean (SD) and groups compared by one-way ANOVA. N, number.

5.4.8.3.2 Hepatic fat content quantified by ^{1}H Spectroscopy

Hepatic fat measurement by proton spectroscopy was limited by technical problems in 3 scans where data were unable to be processed, therefore a total of 37 scans were included in spectroscopy analysis (dutasteride, *N=13*; finasteride, *N=15*; tamsulosin *N=9*). Representative spectra demonstrating low fat fraction and high fat fraction are shown in Figure 5.12 and Figure 5.13 respectively.

Liver spectroscopy results were not normally distributed. Log transformation normalised data however failed on homogeneity of variance, and other transformations tested did not normalise data. Therefore results were analysed by Kruskal-Wallis test. Median (inter-quartile ranges) values were: dutasteride 9.4% (3.59; 23.57), finasteride 4.7% (1.31; 43.20) and tamsulosin 3.4% (1.78; 9.16), and the wide variation in fat fraction results are evident in Figure 5.14. There were no statistically significant differences between treatment groups (p=0.22). There was no baseline measure and relationships between fat fraction, and other potentially relevant covariates (including weight, BMI, body fat, waist circumference, WHR, triglycerides) were not linear, therefore an ANCOVA was not appropriate. Adjustment for these variables by using the fat fraction divided by the variable showed that the data were not significantly different.

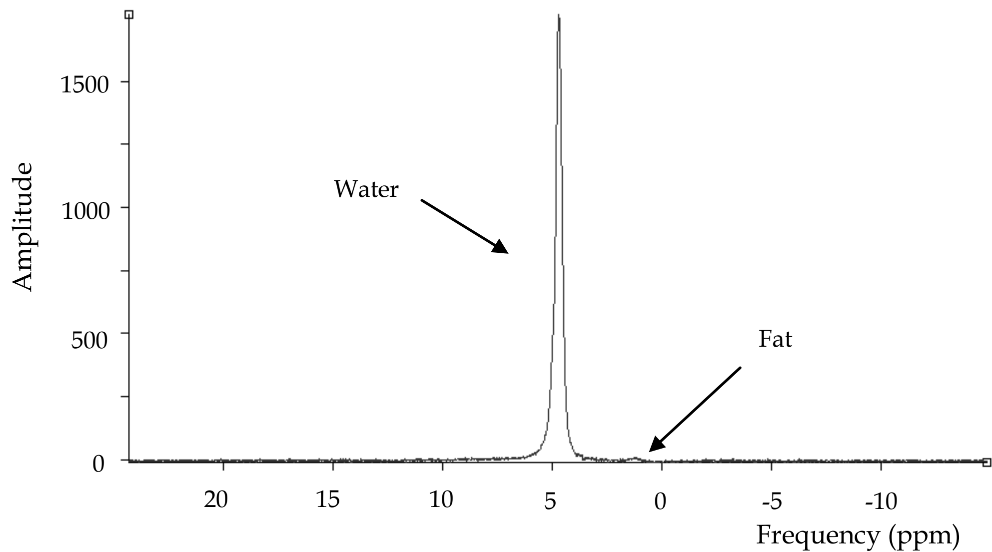

Figure 5.12 Magnetic resonance spectroscopy spectra from subject with low fat fraction, calculated as < 1%. ppm, parts per million.

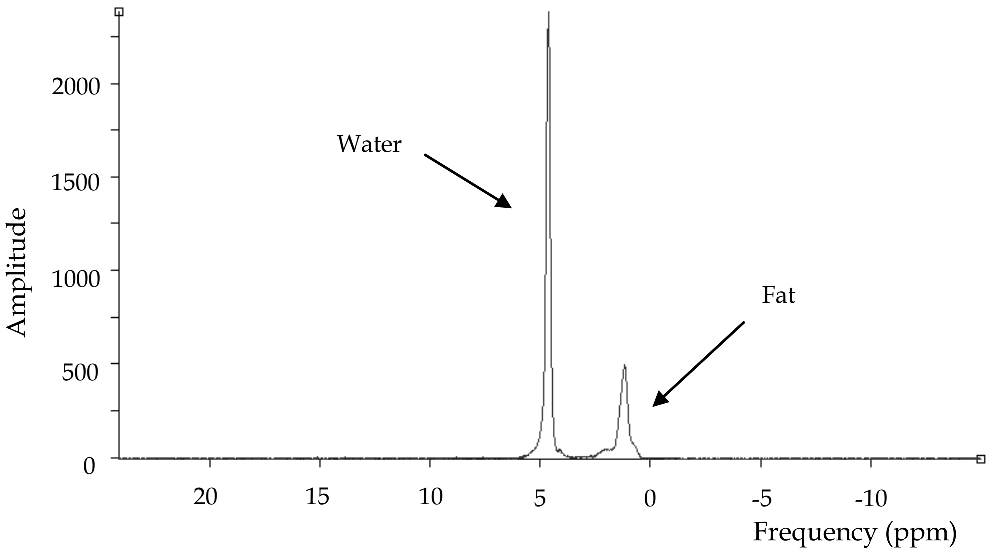

Figure 5.13 Magnetic resonance spectroscopy spectra from subject with high fat fraction, calculated as 27%. ppm, parts per million.

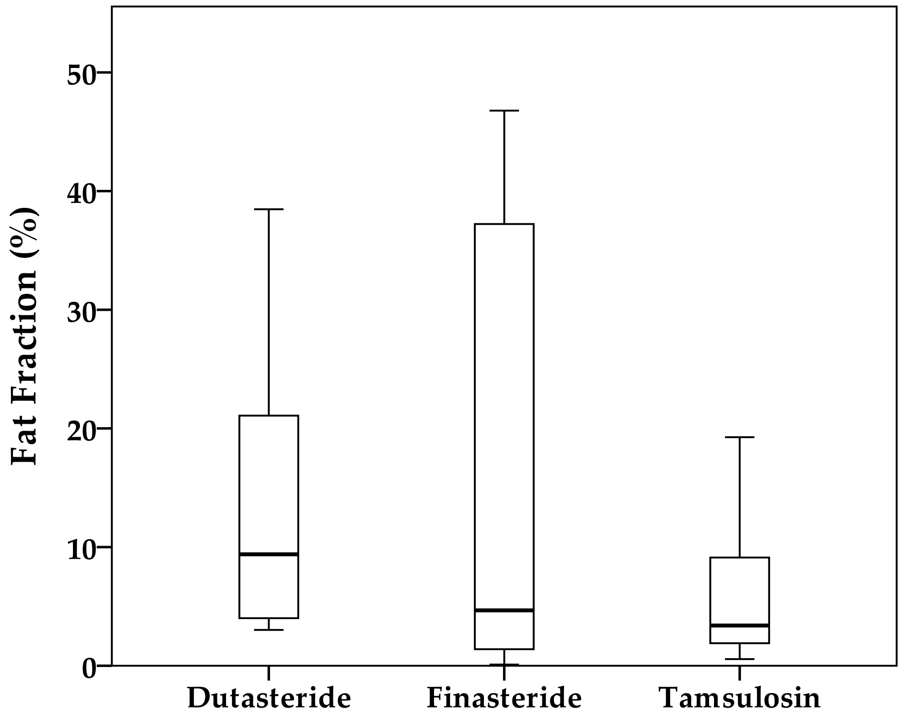

Figure 5.14 Box plot of hepatic fat fractions measured by ^{1}H spectroscopy following three months of dutasteride, finasteride or tamsulosin.

5.4.9 Effect of 5αR inhibition on serum adipokine and cytokine concentrations

The increase in body fat with dutasteride (Section 5.4.8.2) was not associated with an increase in serum adipokines (leptin, adiponectin or resistin), or cytokines (MCP-1 or IL-8), as summarised in Table 5-21. Results adjusted for fat mass did not alter these findings (data not shown).

There was marked variation both within and between groups in leptin concentrations; therefore although leptin appears to increase from baseline with dutasteride, this was not statistically significant. Technical difficulties with the adiponectin and resistin assay and instrument led to many results (especially for adiponectin) having RSDs which were greater than the manufacturer recommended cut-off of 22%. Findings were not altered when data were considered with and without samples with high RSDs (data not shown). MCP-1 did not change with any treatment, although there was a trend towards an increase in serum IL-8 with finasteride.

		Dutasteride	Finasteride	Tamsulosin	**Difference between groups**
	N	*16*	*16*	*14*	
Metabolic adipokines					
Leptin pg/mL	Pre ℞	5865 (6990)	3459 (1844)	6106 (7107)	p=0.32†
	Post ℞	6921 (6259)	3487 (2015)	6398 (7677)	
	Change	*+ 1056*	*+ 27*	*+ 293*	
Adipo µg/mL	Pre ℞	13.4 (8.0)	13.7 (5.8)	16.4 (16.2)	p=0.93
	Post ℞	14.7 (6.4)	14.6 (7.5)	16.7 (9.6)	
	Change	*+ 1.3*	*+ 0.9*	*+ 0.3*	
Inflammatory adipokines and cytokines					
Resistin ng/mL	Pre ℞	23.7 (10.7)	23.3 (11.4)	27.0 (11.4)	p=0.50
	Post ℞	24.0 (9.6)	23.6 (7.0)	29.9 (13.2)	
	Change	*+ 0.4*	*+ 0.2*	*+ 2.9*	
MCP-1 pg/mL	Pre ℞	237 (72.4)	189 (69.6)	274 (110.0)	p=0.33
	Post ℞	238 (83.3)	197 (85.9)	249 (106.9)	
	Change	*+1*	*+8*	*-25*	
IL-8 pg/mL	Pre ℞	5.6 (3.5)	6.0 (4.7)	7.3 (1.7)	p=0.08
	Post ℞	5.6 (3.6)	6.3 (3.8)	6.2 (1.7)	
	Change	*0*	*+0.3*	*-1.1*	

Table 5-21 Serum leptin, adiponectin, resistin, MCP-1, and IL-8 concentrations did not change following three months of either dutasteride, finasteride or tamsulosin. Data are mean (SD). Change from baseline was compared between groups; leptin by †Kruskal-Wallis test and all others by one-way ANOVA. N, number; ℞, treatment; Adipo, adiponectin; MCP-1, monocyte chemoattractant protein 1; IL-8, interleukin 8.

5.4.10 Effect of 5αR inhibition on metabolic and inflammatory gene transcription in subcutaneous adipose

39 of the 46 volunteers had fat biopsies taken before and after treatment, and these were all included in the analysis. All samples had acceptable RNA quality following electrophoretic analysis and were taken forward to reverse transcription and quantitative RT-PCR.

Of the genes tested, only *SRD5A2* and *AKR1C2* were unable to be reliably measured by RTPCR and were therefore not included in the analysis. Error and efficiency results from all transcripts measured are shown in Table 5-22.

Gene	Error	Eff.
ACACA: Acetyl-Coenzyme A carboxylase α isoform 1	0.0138	1.974
PLIN2: Adipocyte differentiation-related protein (Perilipin 2)	0.0250	1.987
ADIPOQ: Adiponectin	0.0315	1.987
AR: Androgen receptor	0.0226	1.725
CYP19A1: Aromatase	0.0759	1.938
DGAT2: Diacylglycerol O-acyltransferase homolog 2	0.0249	1.952
ESR1: Estrogen receptor α	0.0253	1.781
ESR2: Estrogen receptor β	0.0178	1.941
FASN: Fatty acid synthase	0.0176	1.932
GPD2: Glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	0.0375	1.939
GR: Glucorticoid receptor	0.0170	1.794
LIPE: Hormone sensitive lipase	0.0299	1.865
LEP: Leptin	0.0418	2.001
LPL: Lipoprotein lipase	0.0125	1.906
HSD11B1: 11β-Hydroxysteroid dehydrogenase type 1 (11βHSD1)	0.0132	1.882
GPAM: Glycerol-3-phosphate acyltransferase 1 (mitochondrial)	0.0120	1.782
MCP1: Monocyte chemotactic protein 1	0.0113	1.894
PPARG: Peroxisome proliferator-activated receptor γ (PPARγ)	0.0163	1.701
PPARGC1A: PPARγ coactivator 1α	0.0411	2.025
SRD5A1: 5α-Reductase type 1 (5αR1)	0.0139	1.965
SREBF1: Sterol regulatory element binding transcription factor 1	0.0236	2.201
SREBF2: Sterol regulatory element-binding transcription factor 2	0.0287	1.823
UCP 2: Uncoupling protein 2	0.0237	1.858
PPIA: Peptidylprolyl isomerase A (cyclophilin A)	0.0093	1.856
RPL32: Ribosomal protein L32	0.0177	2.084
TBP: TATA-binding protein	0.0091	1.845
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase	0.0242	1.922

Table 5-22 Error and efficiency (Eff.) results from all genes tested. Error was acceptable if <0.05, and efficiency if between 1.7 – 2.2; all genes except aromatase met these criteria.

It was noted that all housekeeping genes tested, and indeed most genes tested, increased in abundance following tamsulosin treatment. The explanation for this finding is unclear. Although not ideal, given this was a consistent finding, it was felt appropriate to combine the best three housekeeping genes (*PPIA, TBP, GAPDH*), as the use of these would normalise gene results from transcripts of interest and still allow comparisons to be made.

Results are summarised in Table 5-23, Table 5-24 and Table 5-25. AR was the only gene to change significantly upon treatment, with mRNA abundance decreased from baseline in both dutasteride and finasteride treated groups, compared to tamsulosin, demonstrated in Figure 5.15. As seen, abundance of AR transcript in dutasteride and finasteride treated groups did not differ from each other. While abundance of other transcripts did not change with treatment, it is recognised that many genes quantified are not solely transcriptionally regulated, therefore non-transcriptional changes would not be detected here.

		Dutasteride	Finasteride	Tamsulosin	**Difference**
	N	*13*	*14*	*12*	
Genes transcribing enzymes for steroid hormone synthesis and metabolism					
SRD5A1 (5αR1)	Pre ℞	0.28 (0.09)	0.28 (0.09)	0.30 (0.11)	p=0.47
	Post ℞	0.29 (0.08)	0.24 (0.10)	0.31 (0.11)	
	Change	*+ 0.01*	*- 0.04*	*+ 0.01*	
HSD11B1 (11βHSD1)	Pre ℞	0.44 (0.29)	0.35 (0.22)	0.52 (0.55)	p=0.58†
	Post ℞	0.28 (0.25)	0.23 (0.12)	0.27 (0.31)	
	Change	*- 0.16*	*- 0.12*	*- 0.25*	
CYP19A1* (Aromatase)	Pre ℞	0.26 (0.25)	0.33 (0.25)	0.24 (0.19)	p=0.998
	Post ℞	0.22 (0.21)	0.29 (0.24)	0.21 (0.13)	
	Change	*- 0.04*	*- 0.03*	*- 0.04*	
Genes transcribing steroid hormone receptors					
AR	Pre ℞	0.34 (0.19)	0.31 (0.12)	0.22 (0.11)	***p=0.045***
	Post ℞	0.22 (0.13)	0.23 (0.11)	0.26 (0.17)	
	Change	*- 0.12*	*- 0.08*	*+ 0.04*	
GR	Pre ℞	0.35 (0.17)	0.34 (0.18)	0.26 (0.12)	p=0.62
	Post ℞	0.34 (0.11)	0.31 (0.14)	0.27 (0.11)	
	Change	*- 0.01*	*- 0.03*	*+ 0.02*	
ESR1 (ERα)	Pre ℞	0.32 (0.13)	0.40 (0.23)	0.32 (0.12)	p=0.40
	Post ℞	0.26 (0.15)	0.32 (0.19)	0.32 (0.15)	
	Change	*- 0.06*	*- 0.07*	*- 0.00*	
ESR2 (ERβ)	Pre ℞	0.32 (0.14)	0.35 (0.13)	0.32 (0.14)	p=0.86
	Post ℞	0.32 (0.22)	0.31 (0.16)	0.29 (0.12)	
	Change	*- 0.01*	*- 0.03*	*- 0.04*	

Table 5-23 Androgen receptor (*AR*) transcript decreased in abundance following 5αR inhibition (details in text and in Figure 5.15). There were no other differences in transcript ratios shown from subcutaneous abdominal adipose biopsies before and after three months of dutasteride, finasteride or tamsulosin. Change from baseline compared between groups by one-way ANOVA. *Standard curve for *CYP19A1* had high error (0.0759). † Analysed by Kruskal-Wallis test. N, number; ℞, treatment.

		Dutasteride	Finasteride	Tamsulosin	**Difference**
	N	*13*	*14*	*12*	
Genes transcribing proteins involved in lipogenesis and lipolysis					
ACACA (Acetyl CoA carboxylase)	Pre ℞	0.20 (0.11)	0.27 (0.16)	0.21 (0.08)	p=0.28†
	Post ℞	0.25 (0.20)	0.24 (0.16)	0.26 (0.16)	
	Change	*+ 0.05*	*- 0.03*	*+ 0.05*	
UCP2 (Uncoupling protein 2)	Pre ℞	0.25 (0.17)	0.24 (0.13)	0.24 (0.14)	p=0.65
	Post ℞	0.29 (0.28)	0.27 (0.14)	0.23 (0.09)	
	Change	*+ 0.04*	*+ 0.03*	*- 0.00*	
FASN (Fatty acid synthase)	Pre ℞	0.29 (0.15)	0.39 (0.28)	0.25 (0.17)	p=0.17†
	Post ℞	0.30 (0.23)	0.28 (0.22)	0.43 (0.47)	
	Change	*+ 0.01*	*- 0.11*	*+ 0.17*	
LIPE (Hormone sensitive lipase)	Pre ℞	0.36 (0.37)	0.44 (0.31)	0.33 (0.18)	p=0.62†
	Post ℞	0.24 (0.15)	0.34 (0.13)	0.32 (0.20)	
	Change	*- 0.12*	*- 0.10*	*- 0.02*	
PLIN2 (Perilipin)	Pre ℞	0.30 (0.16)	0.32 (0.16)	0.33 (0.17)	p=0.97
	Post ℞	0.22 (0.13)	0.25 (0.11)	0.26 (0.13)	
	Change	*- 0.08*	*- 0.07*	*- 0.07*	
PPARG (PPARγ)	Pre ℞	0.46 (0.21)	0.49 (0.17)	0.45 (0.18)	p=0.61
	Post ℞	0.40 (0.20)	0.46 (0.16)	0.48 (0.31)	
	Change	*- 0.07*	*- 0.04*	*+ 0.02*	
PPARGC1A (PPARγ, coactivator 1α)	Pre ℞	0.43 (0.46)	0.38 (0.20)	0.26 (0.09)	p=0.33†
	Post ℞	0.19 (0.08)	0.34 (0.19)	0.25 (0.18)	
	Change	*- 0.24*	*- 0.03*	*- 0.01*	
GPD2 (G3P dehydrogenase)	Pre ℞	0.33 (0.12)	0.30 (0.11)	0.31 (0.09)	p=0.36
	Post ℞	0.38 (0.13)	0.28 (0.09)	0.35 (0.11)	
	Change	*+ 0.04*	*- 0.02*	*+ 0.03*	

Table 5-24 There were no differences in transcript levels shown, from subcutaneous abdominal adipose biopsies before and after three months of dutasteride, finasteride or tamsulosin. Change from baseline compared between groups by one-way ANOVA. † Analysed by Kruskal-Wallis test. N, number; ℞, treatment.

		Dutasteride	Finasteride	Tamsulosin	**Difference**
	N	*13*	*14*	*12*	
Genes transcribing adipokines					
LEP (Leptin)	Pre ℞	0.19 (0.13)	0.19 (0.07)	0.18 (0.14)	p=0.37
	Post ℞	0.17 (0.12)	0.12 (0.07)	0.17 (0.15)	
	Change	*- 0.03*	*- 0.07*	*- 0.01*	
ADIPOQ (Adiponectin)	Pre ℞	0.35 (0.12)	0.40 (0.16)	0.26 (0.12)	p=0.10
	Post ℞	0.32 (0.23)	0.30 (0.16)	0.34 (0.22)	
	Change	*- 0.03*	*- 0.10*	*+ 0.08*	
MCP1	Pre ℞	0.39 (0.20)	0.40 (0.27)	0.48 (0.07)	p=0.47
	Post ℞	0.29 (0.14)	0.39 (0.30)	0.34 (0.22)	
	Change	*- 0.10*	*- 0.01*	*- 0.14*	
Genes transcribing proteins involved in lipid and sterol homeostasis					
DGAT2	Pre ℞	0.22 (0.20)	0.31 (0.20)	0.18 (0.08)	p=0.12
	Post ℞	0.29 (0.23)	0.24 (0.24)	0.32 (0.32)	
	Change	*+ 0.07*	*- 0.07*	*0.14*	
SREBF1	Pre ℞	0.16 (0.14)	0.18 (0.12)	0.13 (0.06)	p=0.28†
	Post ℞	0.17 (0.21)	0.26 (0.29)	0.24 (0.21)	
	Change	*+ 0.01*	*+ 0.08*	*+ 0.11*	
SREBF2	Pre ℞	0.37 (0.15)	0.44 (0.21)	0.36 (0.15)	p=0.27
	Post ℞	0.44 (0.17)	0.40 (0.31)	0.44 (0.27)	
	Change	*+ 0.07*	*- 0.04*	*+ 0.08*	
LPL (Lipoprotein lipase)	Pre ℞	0.32 (0.16)	0.30 (0.10)	0.30 (0.11)	p=0.39
	Post ℞	0.25 (0.15)	0.23 (0.11)	0.31 (0.19)	
	Change	*- 0.07*	*- 0.06*	*+ 0.01*	
GPAM (G3P acyl-transferase)	Pre ℞	0.33 (0.20)	0.28 (0.13)	0.25 (0.13)	p=0.62†
	Post ℞	0.32 (0.22)	0.22 (0.11)	0.27 (0.24)	
	Change	*- 0.01*	*- 0.05*	*+ 0.02*	

Table 5-25 There were no differences in transcript levels shown, from subcutaneous abdominal adipose biopsies before and after three months of dutasteride, finasteride or tamsulosin. Change from baseline compared between groups by one-way ANOVA. † Analysed by Kruskal-Wallis test. N, number; ℞, treatment.

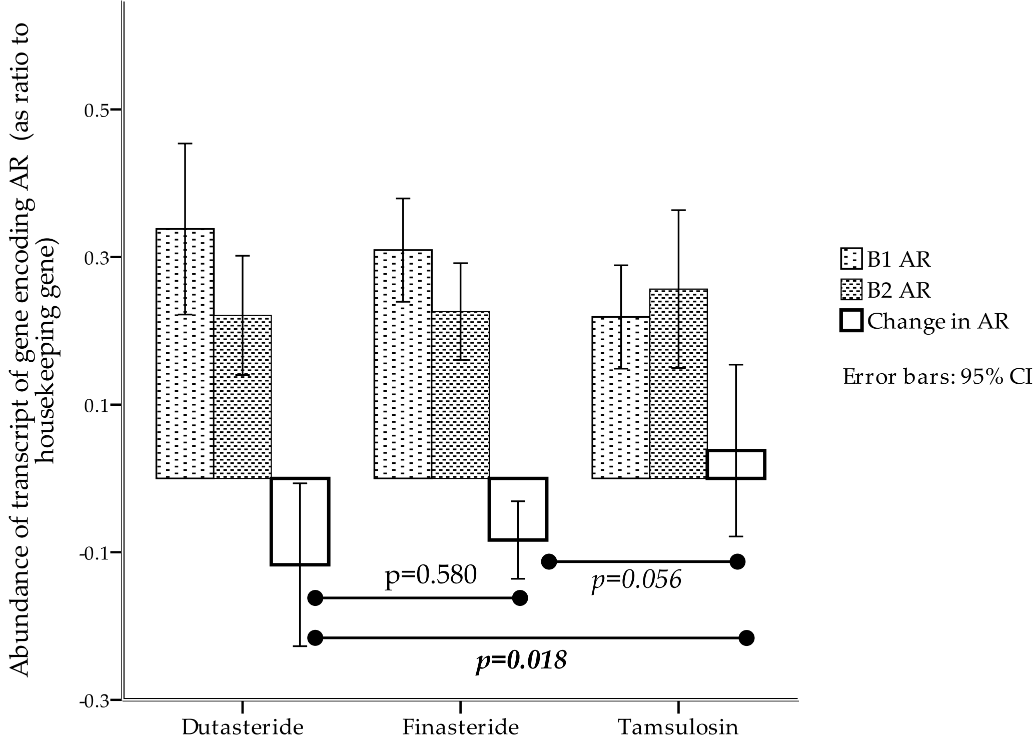

Figure 5.15 Abundance of transcript of gene encoding the androgen receptor (AR) in subcutaneous abdominal adipose before (B1) and after (B2) three months of dutasteride, finasteride or tamsulosin. Abundance decreased following 5αR inhibition. Shown are B1 and B2 means, as well as the change from baseline. Error bars indicate 95% confidence intervals (CI). Pairwise comparisons by one-way ANOVA with LSD post-hoc show mean (95% CI) changes of: dutasteride versus tamsulosin -0.15 (-0.28; -0.03), p=0.02; finasteride versus tamsulosin -0.12 (-0.25; 0.00), p=0.06 and dutasteride versus finasteride -0.03 (-0.16; 0.09), p=0.58.

5.4.11 Effect of 5αR inhibition on circulating lipids

Other than a trend (p=0.08) towards an increase in fasting total cholesterol with finasteride, there were no significant differences between groups. Results are summarised in Table 5-26.

		Dutasteride	Finasteride	Tamsulosin	**Difference between groups**
	N	*16*	*16*	*14*	
Total C mmol/L	Pre ℞	4.41 (0.64)	4.87 (1.01)	5.20 (0.95)	p=0.08
	Post ℞	4.34 (0.78)	4.98 (1.04)	4.93 (1.03)	
	Change	*- 0.07*	*+ 0.11*	*- 0.27*	
HDL-C mmol/L	Pre ℞	1.29 (0.36)	1.28 (0.27)	1.38 (0.65)	p=0.47
	Post ℞	1.27 (0.29)	1.28 (0.24)	1.25 (0.34)	
	Change	*- 0.02*	*0*	*- 0.13*	
LDL-C mmol/L	Pre ℞	2.55 (0.64)	3.03 (1.05)	3.21 (0.76)	p=0.43
	Post ℞	2.57 (0.73)	3.14 (1.02)	3.12 (0.93)	
	Change	*+ 0.02*	*+ 0.11*	*- 0.09*	
TG mmol/L	Pre ℞	1.25 (0.55)	1.21 (0.69)	1.35 (0.74)	p=0.38
	Post ℞	1.10 (0.70)	1.24 (0.65)	1.23 (0.65)	
	Change	*- 0.15*	*+ 0.04*	*- 0.12*	
Total C: HDL ratio	Pre ℞	3.69 (1.17)	3.98 (1.31)	4.25 (1.29)	p=0.69
	Post ℞	3.67 (1.20)	4.08 (1.25)	4.19 (1.36)	
	Change	*- 0.02*	*+ 0.10*	*- 0.06*	

Table 5-26 Fasting serum lipids did not change following three months of dutasteride, finasteride or tamsulosin. Data are mean (SD), and change from baseline is compared between groups by one way ANOVA. N, number; ℞, treatment; C, cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, triglycerides.

5.4.12 Effect of 5αR inhibition on serum liver function tests (LFTs)

Results from LFTs are summarised in Table 5-27. Alkaline phosphatase (ALP) increased from baseline with finasteride and this was statistically significant versus tamsulosin only, with a mean difference of 6.8 U/L (1.58; 12.04), $p=0.01$. There were no other differences between groups. Albumin results are presented in Section 5.4.13.

		Dutasteride	Finasteride	Tamsulosin	**Difference between groups**
	N	*16*	*16*	*14*	
Bilirubin µmol/L (3 – 16)	Pre ℞	13.1 (6.77)	12.7 (5.55)	13.2 (10.47)	p=0.63
	Post ℞	10.4 (3.98)	12.4 (5.11)	11.9 (5.79)	
	Change	*- 2.7*	*- 0.3*	*- 1.3*	
ALT U/L (10 – 50)	Pre ℞	30.0 (13.72)	28.4 (13.05)	28.6 (16.41)	p=0.66
	Post ℞	25.8 (8.72)	28.3 (17.28)	25.4 (10.65)	
	Change	*- 4.2*	*- 0.1*	*- 3.2*	
ALP U/L (40 – 125)	Pre ℞	60.9 (16.81)	72.8 (9.97)	79.3 (20.36)	p=0.04
	Post ℞	58.5 (15.25)	74.1 (12.23)	73.8 (20.51)	
	Change	*- 2.4*	*+ 1.6*	*- 5.5*	
	N	*15*	*16*	*12*	
AST U/L (10 – 45)	Pre ℞	26.9 (7.11)	25.6 (4.72)	28.4 (12.77)	p=0.07
	Post ℞	21.9 (6.49)	27.9 (12.80)	20.6 (5.45)	
	Change	*- 5.0*	*+ 2.3*	*- 7.8*	
GGT U/L (5 – 55)	Pre ℞	22.9 (15.17)	20.1 (11.56)	29.3 (26.23)	p=0.30
	Post ℞	27.2 (15.64)	24.3 (12.39)	26.8 (23.46)	
	Change	*+ 4.3*	*+ 4.2*	*- 2.5*	
AST: ALT ratio	Pre ℞	1.0 (0.36)	1.0 (0.32)	1.1 (0.32)	p=0.73†
	Post ℞	0.9 (0.27)	1.2 (0.99)	1.0 (0.32)	
	Change	*- 0.1*	*+ 0.2*	*- 0.10*	

Table 5-27 Other than alkaline phosphatase (ALP, details in text), serum liver function tests did not change following three months of dutasteride, finasteride or tamsulosin. Data are mean (SD), and change from baseline is compared between groups by one way ANOVA, except AST/ALT ratio compared by †Kruskal Wallis test. N, number; ℞, treatment; ALT, alanine transaminase; AST, aspartate transaminase; GGT, γ glutamyl transpeptidase. Laboratory normal ranges are indicated beneath the name of the test.

5.4.13 Effect of 5αR inhibition on circulating oestradiol, cortisol and steroid hormone binding proteins

There was a trend (p=0.07) towards an increase in serum oestradiol following 5αR inhibition. There was no change in plasma cortisol concentrations, or in the serum binding proteins, albumin, sex hormone binding globulin (SHBG) or corticosteroid binding globulin (CBG). Results are summarised below in Table 5-28.

		Dutasteride	Finasteride	Tamsulosin	**Difference between groups**
	N	*16*	*16*	*14*	
Steroid hormones					
Cortisol nmol/L	Pre ℞	788.5 (236.68)	768.7 (203.49)	817.9 (201.33)	p=0.88
	Post ℞	692.1 (186.20)	688.8 (172.89)	757.3 (157.28)	
	Change	*- 96.4*	*- 79.9*	*- 60.6*	
E2 pmol/L	Pre ℞	81.8 (31.51)	69.2 (29.76)	73.4 (35.17)	**p=0.07†**
	Post ℞	126.7 (67.79)	94.0 (32.01)	80.8 (40.75)	
	Change	*+44.9*	*+24.8*	*+7.4*	
Binding proteins					
SHBG nmol/L	Pre ℞	27.9 (9.01)	24.4 (8.74)	31.1 (11.23)	p=0.25
	Post ℞	27.1 (10.37)	25.1 (9.06)	33.4 (16.02)	
	Change	*- 0.8*	*+ 0.7*	*+ 2.3*	
CBG nmol/L	Pre ℞	988 (172.2)	936 (126.0)	959 (127.4)	p=0.75†
	Post ℞	966 (168.2)	905 (211.6)	929 (156.5)	
	Change	*-22*	*-31*	*-31*	
	N	*15*	*16*	*12*	
Alb g/L	Pre ℞	41.6 (3.07)	43.2 (3.78)	41.2 (4.08)	p=0.26
	Post ℞	38.8 (3.41)	39.9 (2.17)	39.7 (2.46)	
	Change	*- 2.8*	*- 3.3*	*- 1.5*	

Table 5-28 There was a trend towards an increase in oestradiol (E2) following 5αR inhibition. Plasma cortisol concentrations did not change, nor did sex hormone binding globulin (SHBG), corticosteroid binding globulin or albumin (Alb). Data are mean (SD) before and after three months of dutasteride, finasteride or tamsulosin. Change in baseline compared between groups by one-way ANOVA, other than E2 and CBG compared by †Kruskal-Wallis test. N, number; ℞, treatment.

5.5 Discussion

This chapter has presented a double blind randomised controlled study designed to investigate the metabolic consequences of 5αR inhibition in a population of adult men; an area largely neglected in published literature. The principal hypotheses related to the concept that by inhibiting 5αR1 in metabolic tissues, a resulting elevation in local cortisol concentrations would be adverse for metabolic health. Dynamic tests of insulin and glucose homeostasis showed a clear decrease in insulin sensitivity following three months of treatment with the dual 5αR1 and 5αR2 inhibitor dutasteride, versus both the 5αR2 selective inhibitor finasteride and the control group tamsulosin, with data suggestive of impaired peripheral glucose uptake. In addition, body composition was altered with an increase in body fat in the dutasteride group only.

A key objective in this study was to utilise an appropriately powered randomised study design in order to establish causality (Roberts and Torgerson, 1998). In the context of 5αR inhibitors there have been studies investigating some aspects of metabolism (Andriole and Kirby, 2003, Hong et al., 2010), however there was a lack of a well-designed randomised study, including both finasteride and dutasteride, that incorporated sensitive measures of insulin sensitivity. Due to the long half life of dutasteride (5

weeks, (Gravas and Oelke, 2010)), a cross-over study was not possible, and so a parallel group randomised controlled design was selected. The block randomisation method had 18 subjects per block; an adequate block size to prevent allocations being deciphered, whilst also used to ensure an even number of participants in each treatment arm. However, as the study was stopped early treatment groups were slightly unequal in size, though within the recommended acceptable difference of less than half the block size (in this case, 9) (Altman and Dore, 1990). Every effort was made to ensure that potentially important variables (e.g. using the same glucometer) were kept consistent throughout the study. An additional positive feature of the study design was conducting pre- and post-drug study visits in parallel, so factors such as season or medication batches would not differentially affect study groups.

By chance, the three groups in this study appeared to differ at baseline in several measures including age, body fat and insulin sensitivity, with the tamsulosin group in poorer health. In order to ensure that age and metabolic characteristics were approximately equal at baseline, an alternate randomisation method which may have been considered would be 'minimisation' (Treasure and MacRae, 1998), with volunteers randomised according to their insulin sensitivity at baseline. In practice, this would have

entailed determination of the baseline clamp M value (or a surrogate measure such as fasting glucose) and randomisation performed at that stage. Minimisation may have been particularly advantageous in interpretation of post-study MRI results. As the original study protocol included people in a narrower age range (presumably with less heterogeneity in insulin sensitivity), this was not predicted to be an issue at the time of study planning, however in future studies this approach should be considered.

Recruitment was one of the main challenges encountered and necessitated review of study design. Temporarily pursued in parallel was a cross-sectional study with metabolic measures conducted with patients already on 5αR inhibitors; an important consideration given 34% of patients reviewed in BPH clinics were already on 5αR inhibitors. However cross-sectional studies do not minimise confounders by randomisation and are unable to establish causation; given the aims of the study this was not ideal.

Instead several steps were taken to improve recruitment rates and retain the randomised study protocol. Key factors contributing to poor recruitment were the small number of eligible patients attending urology clinics and most eligible participants declining participation. Attempts to widen recruitment access within secondary care were unsuccessful: St John's hospital in

Livingston due to reluctance from clinic staff, and the Borders General Hospital in Melrose due to distance from Edinburgh and low numbers of BPH patients seen. BPH is a chronic disease with the vast majority of patients cared for by GPs; therefore recruitment was widened to include primary care. Patients approached were those with diagnosed BPH on alpha blockers, who did not meet any of the exclusion criteria. While ~400,000 prescriptions for tamsulosin are dispensed in Scotland annually (Scotland, 2011), regional information is not publicly available. In addition, details of co-prescribing or presence of co-morbidities are not collated on a community basis. The nature of this study (i.e. a lack of immediate results pertaining to targets disorders in primary care) meant it proved less attractive for GP practices to invest time and resource, and recruitment of both practices and patients was difficult. However, despite multiple issues, primary care proved a valuable recruitment avenue, providing 15% of volunteers who completed the study. The need for study volunteers to be BPH patients was not an absolute requirement, but the study was initially designed in this manner as it was seen as potentially more acceptable to volunteers to take medication for a disease requiring treatment. However, in the end recruitment of healthy volunteers was the main amendment which increased volunteer numbers.

A single interim analysis was conducted to ensure the initial sample size calculation was not an over-estimation, and the study was stopped early based on these results. The M value was chosen as a surrogate for the primary endpoint, glucose disposal, as results from tracer kinetics were not available at the time. At the final analysis it was seen that results from the M value were almost identical to those of Rd glucose, confirming that this was an appropriate selection. The statistical approach to dealing with the interim analysis was to stop the study with $p<0.016$ (taken from 0.05/3 groups) at interim analysis, and to make no further adjustment at the final analysis. Interim data was analysed by the study team, as the study was conducted without an independent data monitoring committee (DMC), a practice recommended for studies with the described endpoints and duration (Grant et al., 2005). While there would be an argument for independent investigators to conduct an interim data analysis even in small studies such as this, final results were in keeping with interim data, alleviating any concerns about a potential "regression to the truth" with attenuation of treatment effect that can be seen following some interim analyses (Pocock and White, 1999).

Recruitment of healthy volunteers resulted in the inclusion of many volunteers much younger than those typically affected by BPH, and

importantly a great proportion of leaner subjects. Arguably this could influence the physiological effects of the medications, and hence diminish the power of the study. β Cell function declines with age (Ma et al., 2009, Fritsche et al., 2002), however age-related decline in insulin sensitivity may largely be due to changes in body composition, in particular an increase in body fat, rather than age *per se* (Basu et al., 2003, Boden et al., 1993, Ferrannini et al., 1996). In rodent models, animals with increased body weight were more susceptible to the metabolic effects of 5αR disruption or inhibition (Livingstone et al., 2008, Livingstone et al., 2009a); making the results for dutasteride in this human study perhaps even more striking. In addition to metabolism, it was important to establish whether 5αR abundance changes with age. On a population basis, the serum DHT: testosterone (T) ratio remains steady from age 20 onwards (Starka et al., 2009), and indeed in the present study also there was no significant correlation between age and the DHT:T ratio.

One participant, a BPH patient previously on tamsulosin, required rescue medication due to intolerable symptoms upon cessation of tamsulosin at the commencement of the study. Although there are several potential statistical methods for dealing with this (White et al., 2001), no statistical adjustment was made in this study as medications used were not being studied for their

intended therapeutic effects, symptoms were not related to any study endpoints, and tamsulosin was the control group and therefore assumed not to affect study endpoints.

An important consideration in this study was selection of the control group. An active treatment was selected rather than placebo, as the original study design included only BPH patients requiring medical therapy. With the inclusion of healthy volunteers, the study was kept as the original design randomising participants to one of tamsulosin, dutasteride or finasteride. Although utilised as a control, tamsulosin, a selective $\alpha 1_A$ and $\alpha 1_D$ antagonist, could potentially act at extra-prostatic α1 receptors, with principal considerations in this study being the potential effects of tamsulosin on metabolism.

Effects of tamsulosin on insulin sensitivity are not well described. In the present study peripheral insulin sensitivity improved in the tamsulosin group, so while *in vitro* α1 activation increases glucose uptake in muscle cells (Hutchinson and Bengtsson, 2005), the lack of reports on *in vivo* effects of tamsulosin on muscle insulin sensitivity mean it is uncertain but unlikely that tamsulosin would have directly impacted on muscle metabolism and insulin sensitivity in this study. While α1 receptors are expressed in adipose

(Kershaw and Flier, 2004), *in vivo* microdialysis studies in healthy men suggest sympathetic effects on adipose are primarily mediated via β rather than α receptors (Boschmann et al., 2002). While slight improvements in insulin sensitivity may result from α blockade in diabetic patients (Pasanisi et al., 1999, McCarty, 2004), studies use α blockers other than tamsulosin so are not directly comparable. Conversely, there are three case reports in known type 2 diabetics of hyperglycaemia upon commencement of tamsulosin treatment (Borgsteede et al., 2010), however there are many potential confounders in these cases, and certainly in our (non-diabetic) volunteers there was no evidence of hyperglycaemia. In the present study most transcripts quantified from subcutaneous adipose biopsies increased with tamsulosin. The reason for this is not clear. It may reflect an interaction of tamsulosin with RNA stability or the function of other aspects of the assay; without formal assessment it is not possible to confirm the reason.

Tamsulosin is unlikely to have affected hepatic physiology in this study as its effects are not mediated through $\alpha 1_B$ receptors (Lepor, 2007), which are the α1 receptors present in rat liver (Piascik and Perez, 2001), though expression of α1 receptor isozymes in human liver is not described. Tamsulosin did not affect blood pressure or heart rate; an important observation as $\alpha 1_A$ and $\alpha 1_D$ receptors are present in human myocardium and coronary arteries

respectively (Jensen et al., 2009), and are also expressed in large and medium size arteries (in rat) (Piascik and Perez, 2001).

Knowing that 5αR inhibitors take several (usually up to 6) months to give clinically noticeable effects, an alternative study design may have been considered, with the use of open-label tamsulosin where necessary, and the randomisation to one of placebo, dutasteride, or finasteride. This would have reflected clinical practice as usually 5αR inhibitors are not given as first line treatment, not just due to their relatively high cost, but also because most patients require immediate therapeutic benefit from α blockade before combination with 5αR if symptoms persist. In hindsight, especially given that 35 out of the 46 volunteers included in the final analysis were healthy volunteers, the ideal study design would have utilised a placebo as control rather than tamsulosin.

In this study there was an observed improvement in insulin sensitivity with both tamsulosin and finasteride. This was possibly due to the 'Hawthorne effect' (Sedgwick, 2012), a component of the classically described placebo effect, and describes the beneficial response which may occur as a result of participation in research. This is distinct from any effect from the actual drug or intervention administered as part of a study, and rather describes changes

in behaviour as a result of being observed or assessed. Anecdotally from participants this appeared to be the case for most and this remains a possible explanation. However, within group changes were not addressed in this study, as the study question was not whether groups changed from baseline, but rather the comparative change between groups.

With improved recruitment the study was able to progress and go on to address its aims. Insulin sensitivity was the primary endpoint assessed using the gold standard measure, a hyperinsulinaemic euglycaemic clamp (Defronzo et al., 1979). Additional measures included fasting plasma glucose, insulin and C-peptide concentrations.

Fasting glucose did not differ following treatment, and this was in keeping with previous literature (Amory et al., 2007). However it is well recognised that a fasting glucose alone is a very crude index of insulin sensitivity, and indeed this was seen in this study also. The use of a hyperinsulinaemic euglycaemic clamp (Defronzo et al., 1979) allowed detailed assessment of insulin sensitivity and, through combination with deuterated glucose and glycerol tracers, allowed investigation of hepatic and peripheral insulin sensitivity. Deuterated glucose was selected as the tracer, as it cannot be recycled within the gluconeogenic pathway, unlike ^{13}C labeled tracers where

lactate can be reintroduced into glucose synthesis, resulting in an underestimation of glucose turnover (Choukem and Gautier, 2008). The addition of deuteriums at carbon 6 of the glucose molecule ensured non-recycling, thus providing the most accurate estimation of endogenous glucose production (Coggan, 1999). Deuterated glycerol was used to establish insulin sensitivity of adipose tissue. With no re-uptake of glycerol into adipocytes, this was selected as an appropriate marker of lipolysis. The choice and position of label is not thought to be important with glycerol as it is with glucose, and as d5-glycerol does not influence glycerol physiology (Matthews et al., 1991), it was an appropriate choice for tracer molecule.

Compared to both finasteride and tamsulosin, results from hyperinsulinaemic euglycaemic clamps demonstrate a clear decrease in insulin sensitivity with dutasteride. Glucose disposal was ~14% lower after three months of dutasteride. Research into clinically significant changes in glucose disposal has primarily been conducted in trials of oral anti-diabetic agents. Glucose disposal is increased by 13% with metformin (Inzucchi et al., 1998), 35% by the sulphonylurea gliclazide (Vestergaard et al., 1995) and 20-32% with pioglitazone (Miyazaki et al., 2002). There were no diabetic subjects included in the present study; therefore insulin infusion rates used were

lower. However results suggest the decrease in glucose disposal with dutasteride treatment may be clinically relevant.

While there was an overall decrease in insulin sensitivity with dutasteride, the absence of any significant change in EGP during low dose insulin suggests that hepatic insulin sensitivity was preserved following 5αR inhibition. Human liver expresses both 5αR1 and 5αR2 (Russell and Wilson, 1994), and is thought to be the main site of cortisol metabolism by 5αR. 5αR mRNA abundance in liver is increased in morbidly obese patients and shows a positive correlation with fasting serum insulin concentrations (Baudrand et al., 2011). Unfortunately (and surprisingly) Baudrand et al. (Baudrand et al., 2011) made no mention of which 5αR isozyme was quantified. The relative importance of 5αR1 and 5αR2 in human liver is not described. As seen, 5αR inhibition led to a convincing alteration of THF/αTHF ratios; however this change was seen in both dutasteride and finasteride. While the decrease in αTHF was greater with dutasteride, the marked change with finasteride implies that if liver is the main contributor to cortisol 5α reduction, both 5αR1 and 5αR2 must play important roles in this process. It is then perhaps not surprising that the reduction in insulin sensitivity seen with dutasteride only was not able to be localised to the liver as one would have expected finasteride to have exerted at least some of this effect. It may be that the liver

reaches a “threshold” of intra-tissue cortisol after which metabolic effects are seen, but there is no evidence to support this at this stage. Alternatively 5αR1 and 5αR2 may be expressed in different cell-types within the liver, but as yet that is unknown. A further possibility is that there may be a non-linear relationship between hepatic 5αR inhibition and urinary αTHF excretion, with a greater accumulation of cortisol in liver with dutasteride treatment compared to finasteride which is not apparent in urinary ratios. There is no evidence for this in the present context, however proof-of-principle has been demonstrated previously when the decrease in hepatic conversion of cortisone to cortisol with increasing doses of 11βHSD1 inhibitor often does not have a linear relationship with urinary THF concentrations (unpublished data, Professor Brian R Walker).

Hepatic insulin sensitivity was preserved when assessed by glucose kinetics, however hepatic steatosis was also investigated as this was a pathological feature in 5αR1 -/- mice (Livingstone et al., 2009a, Livingstone et al., 2008). Subtle and early changes in liver fat content are difficult to assess in humans, however the use of ^{1}H spectroscopy (Qayyum, 2009) offered the most sensitive investigative tool. Results demonstrate marked heterogeneity in liver fat fraction and no significant differences between treatment groups. In terms of potential peripheral markers of hepatic steatosis, the increase seen in

ALP with finasteride would not be considered clinically significant. While an isolated increase in ALP can be seen in fatty liver (Pantsari and Harrison, 2006) ALP levels were much higher (~ 140 U/L vs. ~ 70 U/L) than in the present study, and incremental change in response to any intervention was not examined, therefore their findings cannot be extrapolated to this study. Transaminases are potential markers of fatty liver disease (Paschos and Paletas, 2009) but similarly to ALP they do not appear to be useful in examination of a small incremental change in healthy volunteers, and indeed no changes were seen in this study.

Body fat increased with dutasteride treatment, though this was not accompanied by measurable changes in either abdominal fat distribution or glycerol tracer kinetics. There was however a decrease in insulin-mediated lipolysis suppression seen as a decrease in NEFA suppression with low-dose insulin infusion during the clamp. The lack of significant change in Ra glycerol and glycerol concentrations may simply reflect difficulties with variability in the analytical method, or biological variability between volunteers. A potential limitation in assessment of transcript abundance in adipose was that adipose sampled was subcutaneous not visceral. With evidence of whole body alterations in adipose insulin sensitivity (demonstrated by changes in NEFA suppression) but no definitive evidence

of alterations in subcutaneous adipose physiology, it may be that effects of 5αR1 inhibition have greater consequence in visceral rather than subcutaneous adipose.

Subcutaneous and visceral abdominal fat mass did not differ between groups. While MRI scanning with the IDEAL sequence (optimised for water and fat separation) (Costa et al., 2008) was utilised, this may still have limitations in detecting small changes in abdominal fat content, particularly if there was not a disproportionate increase in one depot (subcutaneous or visceral) over another. Results were not different whether assessed by single slice at L4/5, or via 5 slices centred on L4/5. Single slice scanning has important resource implications and L4/5 is traditionally considered the landmark of choice (Ross et al., 1992). However several studies suggest scanning at L2/3 or L3 may show greater correlation with total abdominal fat content (Demerath et al., 2007, Han et al., 1997, Abate et al., 1997); the use of a more cranial level in future studies may result in more accurate quantitation and a reduction in sample size required (Demerath et al., 2007). An additional limitation in this study was MRI scanning post-treatment only due to financial and service constraints; attempts to co-vary for the lack of a baseline measure were unsuccessful.

Abundance of transcripts of genes regulating lipid metabolism in subcutaneous adipose did not differ. While studies with paired visceral and subcutaneous adipose sampling demonstrate subcutaneous samples to provide a reliable indicator of metabolic disease (Klimcakova et al., 2011), this may not be the case in studies such as this where a relatively small number of transcripts were measured. Factors influencing depot-specific adipose deposition are complex, however subcutaneous transcripts including DGAT and SREBP1c increase in men with the greatest accumulation of visceral fat following 56 days of overfeeding (Alligier et al., 2013). These transcripts were not different in the present study, however while Alligier et al. only saw a 0.7% increase in body fat %, they did see a significant increase in waist circumference, so perhaps overfeeding leads to a different pattern of fat distribution than 5αR1 inhibition.

Although body fat was increased with dutasteride, food intake and activity levels were not assessed, therefore it is unknown whether these factors were altered. A question that was addressed was whether, either as cause or effect, adipokines were altered in subcutaneous adipose and in serum. Associations with metabolic disease have been described with several adipokines, including leptin (Galletti et al., 2007, Galletti et al., 2012), adiponectin (Arita et al., 1999, Lee et al., 2009), resistin (Reilly et al., 2005, Piestrzeniewicz et al.,

2008), MCP-1 (Kim et al., 2006) and IL-8 (Kim et al., 2006), hence these were selected for quantitation in serum.

There was no change in serum leptin concentrations nor any change in leptin mRNA abundance in subcutaneous adipose; indicating leptin is unlikely to be mediating the increase in body fat. Adiponectin has an inverse association with obesity and metabolic disease, however no such change was seen in this study, suggesting changes are not mediated by changes in adiponectin. The lack of change in serum resistin, MCP-1 and IL-8, and unaltered MCP-1 mRNA abundance in subcutaneous adipose again suggest that metabolic changes following dutasteride treatment are likely not associated with inflammatory changes in adipose tissue.

Glucose disposal at high dose insulin reflects both any residual hepatic glucose production as well as disposal into peripheral tissues. In this study 36/92 (39%) of clamps had a negative calculated endogenous glucose production during high-dose insulin infusion; a result seen when high rates of glucose infusion are required to maintain euglycaemia and likely reflects complete suppression of EGP. The negative values may arise due to incomplete equilibration between the rapidly infused dextrose and the pre-existing tracer (Finegood et al., 1988). One strategy to correct this is through

the addition of d2-glucose to the 20% dextrose infused during hyperinsulinaemia (Finegood et al., 1987); an approach which should be considered in future studies particularly where healthy (often very insulin sensitive) volunteers are studied.

The decrease in glucose disposal during high dose insulin was entirely reminiscent of changes in the M value, indicating that hepatic glucose output was adequately suppressed and glucose disposal was matched by the infusion rates given. This is indicative of predominantly peripheral insulin resistance. Adding further weight to this finding was that in order to maintain euglycaemia following tracer only infusion, those on dutasteride were hyperinsulinaemic and skeletal muscle is known to be the primary insulin-sensitive site of post-prandial glucose uptake (DeFronzo and Tripathy, 2009). The weight-adjusted doses were between 32-59 g d2-glucose during the 90 minute period; amounts approximately equivalent to the 0.5 g/kg dose administered in an IV glucose tolerance test (IVGTT) (Bingley et al., 1992), though given continuously rather than as a bolus. A maximum IVGTT dose is usually 35 g, however effects on insulin concentrations plateau at ~ 20 g of glucose (Bingley et al., 1992) therefore variations in amount in the current study would be unlikely to have a material difference in insulin response. Further confirmation of peripheral insulin resistance was

the expected pancreatic response with increases in fasting C-peptide, HOMA-IR and a trend towards an increase in insulin concentrations.

The finding of a predominantly muscle rather than hepatic phenotype was unexpected given the background animal data which demonstrated hepatic insulin resistance and fatty liver in 5αR1 knockout mice and pharmacologically treated Zucker rats (Livingstone et al., 2008, Livingstone et al., 2009a). Skeletal muscle biopsies were therefore not included in the study design, nor was skeletal muscle ^{1}H spectroscopy, so it is unknown whether direct indicators of aberrant glucose physiology or lipid accumulation within skeletal muscle may be present in those treated with dutasteride.

Skeletal muscle insulin resistance is thought to be the initial pathology preceding the development of type 2 diabetes (DeFronzo and Tripathy, 2009). In response to high carbohydrate meals, young insulin resistant compared to insulin sensitive men (matched for age, weight, BMI and activity levels) demonstrate significantly impaired muscle glycogen synthesis and divergence of carbohydrate substrate towards hepatic lipogenesis and triglyceride synthesis (Petersen et al., 2007). This was in the absence of changes in visceral fat or plasma adipokine concentrations (Petersen et al.,

2007). Their findings suggested impaired muscle insulin sensitivity preceded hepatic and visceral adipose insulin resistance, and increased susceptibility has been demonstrated in the elderly (Flannery et al., 2012). Furthermore, the effects of skeletal muscle insulin resistance on hepatic lipogenesis are, at least in part, reversed with exercise (Rabol et al., 2011). In the present study there was no change in fasting triglycerides, and muscle glycogen synthesis was not assessed. It is possible however that the skeletal muscle insulin resistance demonstrated may have preceded changes in hepatic insulin sensitivity with a longer duration of dutasteride treatment.

Muscle insulin sensitivity has not been reported in 5αR models, though it is possible that this was also present but not studied. An alternate possibility is that there may be important inter-species differences in the early pathophysiology and development of the metabolic syndrome. In rats, hepatic insulin resistance is seen prior to muscle insulin resistance in response to high fat feeding (Kraegen et al., 1991). The sequence of tissue-specific effects in the development of insulin resistance in response to 5αR genetic manipulation or pharmacological inhibition is not understood at present and is the subject of ongoing research.

Determining the mechanism behind the decrease in skeletal muscle insulin sensitivity with dutasteride treatment principally involves determining the site of pathology (whether localised within muscle or a paracrine effect), and determining which steroid hormone(s) may be mediating these effects. Expression and importance of 5αR in skeletal muscle is not as well studied as other tissue types. 5αR1 mRNA and protein (Pollanen et al., 2011, Yarrow et al., 2012) are present in human skeletal muscle, though *in vitro* activity was not detected (Thigpen et al., 1993). There are low levels of 5αR2 mRNA expression (Pollanen et al., 2011) but not protein or activity (Thigpen et al., 1993). 5αR3 protein is also present in human skeletal muscle (Godoy et al., 2011, Yarrow et al., 2012).

The presence of 5αR protein in muscle does not necessarily mean it plays an important role in normal physiology. Muscle biopsies from people with neuromuscular disorders (n=*24*) and controls (n=*7*) had no detectable 5αR activity, though did have evidence of DHT metabolism by 3αHSD (Stuerenburg and Schoser, 1999). Others have reported detectable, but likely (in comparison to adipose) negligible conversion of testosterone to DHT *in vivo* (Longcope and Fineberg, 1985). Whether the post-exercise increase in muscle 5αR1 mRNA abundance in rats (Aizawa et al., 2010) is mirrored following exercise in humans is not known.

The role of 5αR in muscle in the metabolism of other 5αR substrates, including glucocorticoids, is unknown, but these steroids play well established roles in regulating muscle insulin sensitivity. Given the lack of consistent evidence to date of the importance of 5αR in muscle with respect to androgen metabolism, it is possible that other substrates are of greater importance.

With potentially little 5αR activity in skeletal muscle, effects seen may be paracrine due to signalling molecules, such as adipokines or changes in other steroid hormones. Skeletal muscle itself produces chemokines including MCP-1 and IL-8 (Wei et al., 2008, Pedersen and Akerstroem, 2007, Boyd et al., 2006). While these were measured as 'adipokines' in the present study, of course circulating concentrations could reflect muscle synthesis as well. With no changes demonstrated, it is likely that chemokines and inflammatory changes within muscle are not mediating the effects seen, and rather steroid hormones may be playing a greater role, and this is discussed further below.

Decreased adipose insulin sensitivity following dutasteride treatment, with an increase in circulating NEFAs, could itself result in a decrease in skeletal muscle insulin resistance. Insulin action in muscle is not impeded directly by NEFAs, rather they are converted first to intramyocellular triglycerides (seen

to increase on ^{1}H spectroscopy) then to long chain acyl CoA which impairs insulin signaling (Boden et al., 2001). In addition, other triglyceride metabolites such as diacylglycerol and ceramide may also impair insulin sensitivity in skeletal muscle, through mechanisms which may include an increase in serine phosphorylation of IRS-1 (DeFronzo and Tripathy, 2009). Circulating NEFAs and intramyocellular ectopic fat accumulation have a strong negative correlation with peripheral glucose uptake in lean (Krssak et al., 1999) and obese (Sinha et al., 2002) non-diabetics. In addition, NEFAs are also associated with a decrease in muscle glycogen synthesis (Griffin et al., 1999, Phillips et al., 1996). A seemingly paradoxical increase in intramyocellular lipid in athletes (Goodpaster et al., 2001) is thought to serve as an energy source for exercise, but in others excess free fatty acids in the circulation are stored as lipid in muscle and drive insulin resistance. While correlation of muscle lipid with insulin sensitivity is extensively described, causation is yet to be established, and while an interesting possible mechanism of action in the present study, it is unclear how likely this possibility may be.

The primary site of the decrease in insulin sensitivity with dutasteride treatment appears to be peripheral, with evidence of impaired insulin sensitivity in both adipose and skeletal muscle. Mediators and mechanisms

underpinning the changes seen are difficult (and likely impossible) to establish completely, however key likely mediators are glucocorticoids and/or sex hormones. In the study presented here, it is not possible to dissect these changes although this may be investigated further in animal models. In rodents gonadectomy may be utilised as a means of excluding (or at least diminishing) androgen-mediated effects, while adrenalectomy can be used to exclude effects of endogenous glucocorticoids. While very useful research tools, these approaches do have limitations: gonadectomy exposes the animal to less testosterone and not just less DHT (therefore cannot directly be extrapolated to studies of 5αR inhibition), and with adrenalectomy loss of mineralocorticoid and adrenal medullary hormones may confound interpretation. Despite these limitations however, results in castrated Zucker rats treated with 5aR inhibitors demonstrated persistence of the insulin resistance phenotype, along with steatosis even after gonadectomy; this suggested that effects were glucocorticoid mediated (Livingstone et al., 2009a). Finasteride in rodents is a dual 5αR1 and 5αR2 inhibitor, and rat liver only expresses 5αR1 (Normington and Russell, 1992). Therefore hepatic effects of finasteride in rodents are likely to be the result of 5αR1 inhibition.

In the present human study surrogate markers of androgen, glucocorticoid and oestrogen function were used in an effort to gain insight into the

mechanisms underpinning the metabolic effects seen. Measurement of steroid hormone concentrations and urinary metabolites demonstrated that both 5αR inhibitors had no effect on circulating cortisol, however did have similar effects in reducing serum DHT, as well as 5α glucocorticoid and androgen metabolites in urine. Testosterone did not increase significantly, however there was a trend towards an increase in oestradiol in the dutasteride group.

The hormones mediating the metabolic effects seen with dutasteride treatment are likely to be either androgens or glucocorticoids. A decrease in serum DHT was seen with 5αR inhibition, and this may contribute to a phenotype of skeletal muscle insulin resistance. In cultured skeletal muscle cells from rat the addition of testosterone and DHEA increases GLUT4 expression and translocation, and this process is inhibited with finasteride (Sato et al., 2008); demonstrating a DHT-dependent mechanism of action. As finasteride in rodents is a dual 5αR1 and 5αR2 inhibitor, this would be comparable to dutasteride in humans. *In vivo,* severe hypogonadism (testosterone <8 nmol/L) is associated with insulin resistance (Tajar et al., 2012), however the implications of an isolated decrease in DHT are not known.

While decreased circulating DHT may contribute to the peripheral insulin resistance seen with dutasteride, the main difficulty with this concept is the similarity in degree of serum DHT inhibition with both 5αR inhibitors. Results demonstrated neither the extent of DHT suppression, nor the distinction between finasteride and dutasteride expected. DHT concentrations seen at baseline were within normal ranges described by LC-MS/MS methods, and as expected lower than (relatively non-specific) RIA methods (Shiraishi et al., 2008), supporting the use of this analytical assay. DHT concentrations were expected to be suppressed by ~70% with finasteride and ~95% with dutasteride, by 4 weeks of treatment, analysed by GC-MS (Clark et al., 2004). In contrast to what was expected given the limits of quantitation in the present assay (Chapter 4), DHT was quantifiable in most volunteers even after 5αR inhibition, both dutasteride and finasteride suppressed DHT by approximately 50%, and perhaps more importantly, they did not differ from each other. While study tablets were blinded with an external coating, other studies use a similar approach. Extraction method and efficiency were not described by Clark et al. (Clark et al., 2004), and 95% confidence intervals were not able to be calculated from data presented. In another study (Wurzel et al., 2007), three months of dutasteride treatment decreased serum DHT concentrations by 77.5% compared to placebo (95% CI: of 55.4 – 100%); however they did not do a parallel assessment with

finasteride treatment, but this does demonstrate differences and variability in response to dutasteride, and results in the present study would be more comparable to these findings. It could perhaps be argued that Clark et al. (Clark et al., 2004) studied 5αR inhibition only in BPH patients, and although the prostate contributes little DHT to the circulation normally (Toorians et al., 2003), with larger prostates and potentially greater prostatic contribution to circulating DHT this may render BPH patients more sensitive to the effects of 5αR inhibition. It is difficult to pin down the exact reason for the unexpected similarity in androgen effects between dutasteride and finasteride seen in this study, though this implies that a reduction in circulating DHT was likely not the mechanism behind the marked differences in metabolic consequences.

The decrease in AR transcript expression in subcutaneous adipose biopsies is likely a consequence of reduced signaling by DHT, and this supports the efficacy of the 5αR inhibitors administered and suggests they accessed adipose. DHT is known to upregulate adipose AR protein *in vitro* (Dieudonne et al., 1998), however down-regulation of AR in response to a decrease in DHT has not been described. While a decrease in androgen action in adipose is associated with insulin resistance (McInnes et al., 2012), the decrease in AR in the present study was seen with both dutasteride and

finasteride, suggesting that this is not related to which isozyme of 5αR is targeted and that down-regulation of androgen action in adipose is not mediating the metabolic differences seen. AR is present in skeletal muscle (Sinha-Hikim et al., 2004), however whether this is altered with 5αR inhibition is unknown.

There was no change in SHBG concentrations seen as a result of 5αR inhibition with either dutasteride or finasteride, which might have led to different delivery of bioavailable steroid, and this is keeping with previous studies (Jaffe et al., 1994, Iranmanesh and Veldhuis, 2005). Together with an unchanged albumin, the lack of effect on SHBG suggests that androgen metabolic clearance was unaltered (Petra et al., 1985), and therefore unlikely to account for metabolic effects seen.

There was no change in circulating cortisol concentrations following dutasteride treatment (due to adaptation by the HPA axis, Chapter 6), therefore any glucocorticoid mediated effect on muscle would need to be either via an increase in cortisol within muscle, or effects on other tissues which then signal to decrease muscle insulin sensitivity. Excess tissue glucocorticoids are recognised to affect muscle insulin sensitivity. In primary culture, dexamethasone pre-treatment decreases insulin-mediated glucose

uptake (Gathercole et al., 2007), whereas in rat skeletal muscle glucocorticoids do not affect insulin receptor number or function (Block and Buse, 1989) but do impede GLUT4 translocation (Dimitriadis et al., 1997). Increased GRα expression in human skeletal muscle cells is associated with the metabolic syndrome (Whorwood et al., 2002). GRα expression in subcutaneous adipose was unchanged in the present study, however any changes in transcript abundance in skeletal muscle are unknown. Urinary glucocorticoid metabolites following treatment suggest a reasonable degree of 5αR metabolism of cortisol is mediated by 5αR2, presumably hepatic 5αR2. Therefore, if metabolic changes seen are related to cortisol metabolism, this is perhaps indicative of a 'threshold' of 5αR inhibition which goes on to cause metabolic derangement or alternatively cell-specific gene expression in the liver. It is otherwise difficult to explain why relatively small changes in glucocorticoid metabolite excretion between the two groups could account for such differing results in insulin sensitivity.

Finally the possible consequences of 5αR1 inhibition on oestradiol signalling was considered. In the present study, there was a trend towards an increase in oestradiol with dutasteride, but not finasteride. This may reflect increased metabolism of testosterone by aromatase in the context of 5αR inhibition. Another possible mechanism is that 5α-reduced androgens are thought to be

natural aromatase inhibitors (Iqbal et al., 1983), therefore the decrease seen in DHT with 5αR inhibition may increase aromatase activity. Previous literature is mixed in reports of effects of 5αR inhibition on oestradiol concentrations. Dutasteride in combination with testosterone increases oestradiol after both 3 and 6 months of treatment (Page et al., 2011a), however a similar effect was seen with testosterone alone, therefore in this case may simply reflect additional aromatase substrate. After a year of either dutasteride or finasteride, there is no change in circulating oestradiol concentrations (Amory et al., 2007), so it is unclear whether the increase seen in the present study is perhaps a transient phenomenon.

Increased oestradiol following dutasteride treatment may mediate some of the metabolic changes seen in this study. Higher endogenous oestradiol concentrations in men are associated with an increased risk of developing diabetes, independent of degree of adiposity (Vikan et al., 2010, Phillips et al., 2003). Paradoxically, in hypogonadal obese patients with type 2 diabetes oestradiol concentrations are decreased (Dhindsa et al., 2011). As with glucocorticoids, it appears with oestradiol also that any aberration from the norm is associated with metabolic disease, rather than change in one direction only. While associations have been reported, causation between oestrogens and metabolic disease in men are not clear, therefore their

possible role in this study remain unconfirmed. An important factor in interpretation of all oestradiol concentrations (including in the present study) is that the analytical method employed is almost always an immunoassay. There is some way to go before difficulties in measuring oestrogens via mass spectrometry are overcome, but perhaps this would aid in establishing the exact effects of 5αR inhibitors on circulating oestradiol concentrations.

This chapter has focused on the roles of 5αR1 and 5αR2, and the consequences of inhibiting these isozymes. However these are not the only isozymes of 5αR; there is at least one other, identified as 5αR3 (Uemura et al., 2008). At this stage, little is known about this isozyme and its importance *in vivo*. While the hypothesis in the present study was that the differential effects seen between dutasteride and finasteride therapy were mediated by their differential effects on 5αR1, it is also possible that changes seen are in fact (in part or wholly) mediated by 5αR3, or another as yet unidentified isozyme. With widespread distribution, particularly in liver, kidney, skeletal muscle, prostate and skin (Godoy et al., 2011), 5αR3 may play an important role in steroid hormone metabolism. The expression of 5αR3 in adipose is not known. Dutasteride is a known 5αR3 inhibitor (Mohler et al., 2011), though details of inhibition are not described. Finasteride is not thought to inhibit 5αR3 (Mohler et al., 2011). Deciphering the role of 5αR3 in the study findings

presented would be an interesting avenue of further research, though considerable work is yet to be done to establish the importance of 5αR3, the role of 5αR3 in androgen and glucocorticoid metabolism (5αR3 metabolism of glucocorticoids has not yet been described), and the actions of the 5αR inhibitors (at clinical doses) against this isozyme.

In conclusion, this chapter has described a double-blind randomised controlled study investigating the metabolic consequences of 5αR inhibition in men. Challenges, most notably poor recruitment, were overcome and the study was completed with 46 men included in the final analysis. Results demonstrate a clear decrease in insulin sensitivity and increase in body fat following three months of dutasteride treatment. The lack of such changes with finasteride implies that changes are likely mediated by 5αR1. The decrease in insulin sensitivity appeared most likely to be originating from skeletal muscle. Deciphering the exact mechanisms for these results are subject of ongoing work, however it has been clearly demonstrated for the first time in a human population that inhibition of 5αR with dutasteride increases body fat and impairs insulin sensitivity. These findings may have important implications for patients prescribed long-term dutasteride for benign prostatic hyperplasia.

Chapter 6: The effects of inhibition of 5α-reductases on the hypothalamic-pituitary-adrenal axis

6.1 Introduction

The HPA axis regulates circulating cortisol concentrations, and adaptation of the axis in response to negative feedback allows an appropriate physiological response to stress. However, subtle alterations in the HPA axis, whether up-regulation or down-regulation, have been associated with presence of, or predisposition to a number of disease processes. Dysregulation of the HPA axis is associated with stress and psychiatric disease (McEwen, 1998, Bradley and Dinan, 2010). Impaired glucocorticoid clearance is associated with down-regulation of the HPA axis in chronic liver disease (McNeilly et al., 2010). Activation of the HPA axis may be insufficient for patient needs during critical illness (Arafah, 2006). The metabolic syndrome has been associated with relatively elevated circulating cortisol concentrations and potential HPA axis impairment (Phillips et al., 1998, Phillips et al., 2000, Bjorntorp et al., 1999).

Alterations in glucocorticoid regulation may influence the HPA axis peripherally, and also directly at sites of HPA axis feedback. Enzymes regulating tissue glucocorticoid concentrations include 11βHSDs (Walker and Andrew, 2006) and 5αRs (Russell and Wilson, 1994). Changes in glucocorticoid clearance by 5αR have been associated with HPA axis dysfunction in metabolic disease. Increased activity of 5αR is associated with obesity and polycystic ovarian syndrome (Vassiliadi et al., 2009, Andrew et

al., 1998, Reynolds et al., 2001, Rask et al., 2002). The increased 5αR activity seen in PCOS has been hypothesised to feedback and drive increased ACTH and subsequent normalisation of circulating cortisol, and an associated increase in adrenal androgen synthesis (Stewart et al., 1990, Vassiliadi et al., 2009). In obesity increased 5αR activity may be a compensatory response which results in maintenance of hepatic insulin sensitivity.

5αRs are widely distributed in sites which may directly or indirectly affect the HPA axis. In humans, 5αR1 mRNA and enzyme activity have been detected in hypothalamus (Thigpen et al., 1993), though not in pituitary (Russell and Wilson, 1994, Thigpen et al., 1993). In the human adrenal no 5αR1 mRNA was detected, however in conditions optimised for 5αR1, low levels of activity were present (Thigpen et al., 1993). In contrast, 5αR2 is not known to be expressed in human hypothalamus, pituitary or adrenal (Thigpen et al., 1993). In addition, both 5αR1 and 5αR2 are widely expressed in metabolic and reproductive tissues respectively, and both isozymes are expressed in liver (Russell and Wilson, 1994). In rodents, which are often used as model systems, there is 5αR1 mRNA and protein in hypothalamus, pituitary and adrenal, while 5αR2 activity has been detected in hypothalamus and pituitary, both 5αR2 mRNA and activity are detected in the adrenal (summarised in Table 1-1).

In principle, a peripheral decrease in 5αR activity would decrease glucocorticoid clearance, however the tendency for cortisol to rise in the circulation would be fully compensated for by a functioning HPA axis. However, with 5αR also expressed in the brain, a decrease in 5αR activity in the brain could increase cortisol concentrations at HPA feedback sites, which may alter the set point of the HPA axis and hence cause a sustained reduction in plasma cortisol concentrations. As is seen with adrenocortical atrophy following pituitary disease (Reimondo et al., 2008), a compensatory downregulation of the HPA axis following 5αR inhibition, and associated decreased cortisol clearance, may lead to decreased adrenocortical responsiveness to ACTH and potentially hinder the stress response. Given the expression pattern of the 5αR isozymes it was hypothesised that 5αR2 inhibition with finasteride may have some effect on the HPA axis, however dual 5αR1 and 5αR2 inhibition with dutasteride would likely have a greater effect. Findings in animal models support the hypothesis: in adrenalectomised 5αR1 -/- mice clearance of infused corticosterone is impaired and corticosterone responses to ACTH or to stress are reduced (unpublished).

In humans, studies of the effects of finasteride on the HPA axis show no effect on basal cortisol concentrations (Rittmaster et al., 1994, Lewis et al., 1997, Uygur et al., 1998), while response to ACTH (250 μg) has been shown

to be unaffected by some (Rittmaster et al., 1994), but decreased by others (Fruzzetti et al., 1994). The effects of dutasteride on the HPA axis are unknown.

Given the lack of randomised studies with dynamic testing of the HPA axis in response to both finasteride and dutasteride, this study was undertaken. The study described in Chapter 5 was designed principally to investigate the effect of 5αR inhibitors on metabolism and as a secondary endpoint, effects of 5αR inhibition on the HPA axis were also investigated.

6.1.1 Hypothesis and Aims

6.1.1.1 Hypothesis

Pharmacological inhibition of 5αRs in the periphery impairs glucocorticoid clearance, enhances negative feedback and hence causes relative adrenal insufficiency.

Pharmacological inhibition of 5αRs in HPA negative feedback sites enhances negative feedback and lowers circulating cortisol concentrations.

The extent of HPA axis dysfunction, both peripherally and centrally, is greater when both isozymes of 5αR are inhibited than 5αR2 alone.

6.1.1.2 Aims

Establish whether pharmacological inhibition of 5αR leads to dysregulation of the HPA axis in men.

Establish whether there are differing effects between the effects of finasteride (a 5αR2 inhibitor) and dutasteride (a dual 5αR1 and 5αR2 inhibitor).

6.2 Methods

This study was conducted as per the methods described in Chapter 5. Aspects of the study relevant to the HPA axis will be briefly summarised here also.

6.2.1 Study design

Study design was a double-blind randomised controlled study, as described in Chapter 5.3.1.

6.2.2 Study protocol

The study protocol is described in detail in Chapter 5.3.4.

6.2.2.1 Study day A

This was the screening visit, as described in Chapter 5.3.4.3.

6.2.2.2 Study day B

Basal measures of the HPA axis were conducted on this visit.

Volunteers returned 24 hour urine collections (described in Chapter 5.3.5.8, processing described in Chapter 5.3.7) and saliva collections (collection described in Chapter 5.3.5.9, processing described in Chapter 5.3.7).

Fasting samples were taken at ~ 8 am; these were plasma for analysis of cortisol, and serum for analysis of DHEAS and androstenedione. Samples were collected as described in Chapter 5.3.5.6.1, and processed as described in Chapter 5.3.7.

6.2.2.3 Study day C

This study visit involved dynamic testing of the HPA axis.

6.2.2.3.1 Low dose dexamethasone suppression test

The evening prior to the visit, patients were asked to take dexamethasone (250 µg) orally at 10 pm and then remain fasted overnight and attend at 7:30 am the following morning. A 20 gauge cannula was inserted in the antecubital fossa and half an hour later a blood sample was taken for measurement of plasma cortisol.

6.2.2.3.2 Low dose short Synacthen test

Following sampling for the dexamethasone suppression test, Synacthen (1 µg, prepared as described in Chapter 5.3.6.6) was administered IV, and flushed with normal saline (0.9%, 10 mL). Sampling for plasma cortisol concentrations was performed at 20, 30, 40 and 60 minutes after Synacthen was given.

Samples during visit C were collected as described in Chapter 5.3.4.5.

6.2.2.4 Treatment

Volunteers were randomised to three months of dutasteride, finasteride or tamsulosin, as described in Chapter 5.3.4.6.

6.2.2.5 Reassessment post treatment with repeat study days B and C

Repeat visits B and C, including repeat urine and saliva collections, were conducted following the treatment phase of the study.

6.2.3 Sample processing

Samples were processed as described in Chapter 5.3.7 .

6.2.4 Data analysis

Statistical methods were as described in Chapter 5.3.8.7. In addition, the Sign test was used to compare time to peak cortisol following Synacthen administration.

Plasma free cortisol was calculated using Coolens' equation (Coolens et al., 1987):

$$U^2K(1+N) + U(1+N+K(G-C)) - C = 0$$

where U is unbound (free) cortisol (μmol/L); K is the affinity of CBG for cortisol at 37 °C; N is the ratio of albumin bound to free cortisol (the conventional value of 1.74 was used); G is plasma CBG level (μmol/L); and C is plasma cortisol level (μmol/L).

The following equations were used for analysis of urinary steroids:

Equation 1.2.4.1

$$Total\ glucocorticoids = \alpha THF + \beta THF + THE$$

Equation 1.2.4.2

$$Total\ androgens = androsterone + aetiocholanolone$$

Equation 1.2.4.3

$$11\beta HSD\ activity = \frac{(\alpha THF + \beta THF)}{THE}$$

Equation 1.2.4.4

$$11\beta HSD2\ activity = F/E$$

Ulick's A ring reductase quotients (Ulick et al., 1992) (relative 5α- and 5β-reduction of cortisol):

Equation 1.2.4.5

$$X = \frac{F}{\alpha\ THF}$$

Equation 1.2.4.6

$$X = \frac{F}{\beta\ THF}$$

Balance of 5αR and 5βR in glucocorticoid metabolism:

Equation 1.2.4.6

$$X = \frac{\beta THF}{\alpha THF}$$

Balance of 5αR and 5βR in androgen metabolism:

Equation 1.2.4.7

$$X = \frac{Aetiocholanolone\ (\beta)}{Androsterone\ (\alpha)}$$

6.3 Results

All 46 men included in the final analysis provided samples for measurement of hormones in urine, saliva, and morning plasma/serum. 45 Men participated in dynamic testing of the HPA axis.

6.3.1 Effects of 5αR inhibition on daily production of glucocorticoids and androgens

Daily production rates of glucocorticoids and androgens were assessed by their amounts in 24 hour urine collections.

Changes in urinary glucocorticoids are shown in Table 6-1. There was a trend ($p=0.06$) towards a decrease from baseline in amounts of total glucocorticoids following 5αR inhibition. A decrease from baseline in the amount of αTHF with both dutasteride and finasteride was seen, without a compensatory increase in 5β-reduced metabolites.

Changes in urinary androgens are shown in Table 6-2. Both 5αR inhibitors suppressed production of androsterone, the 5α-reduced metabolite of androstenedione. In contrast to glucocorticoid metabolism, with 5αR inhibition there was a compensatory increase in androgen metabolites formed by 5β-reduction (measured as an increase in aetiocholanolone) with

both 5αR inhibitors. There was a significant decrease in total androgens with finasteride only.

Urinary steroid ratios reflecting A ring metabolism are shown in Table 6-3. Both glucocorticoids and androgens show a decrease in 5α- compared to 5β-metabolism, following 5αR inhibition. There was no change in the F/βTHF ratio.

Indices of 11βHSD activity are shown in Table 6-4. There was no evidence of change in 11βHSD2 activity (F/E ratio). There was however a decrease in the index of global metabolism of cortisol by 11βHSDs, seen as a decrease in the (βTHF+αTHF)/THE ratio.

		Dutasteride	Finasteride	Tamsulosin	**Δ Dutasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Finasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Dutasteride vs. Δ Finasteride** Mean (95% CI)
	N	*16*	*16*	*14*			
Total GC μg/day	Pre ℞	9166 (2486.7)	9522 (4145.7)	9194 (3007.5)	**Difference between groups: p=0.06**		
	Post ℞	7147 (2500.0)	6998 (2460.4)	9228 (3590.4)			
	Change	*-2019*	*-2524*	*+34*			
F μg/day	Pre ℞	118.73 (44.76)	143.94 (52.62)	143.36 (45.44)	Difference between groups: p=0.71		
	Post ℞	123.19 (44.11)	135.01 (54.40)	136.74 (39.35)			
	Change	*+4.47*	*-8.93*	*-6.61*			
5αTHF μg/day	Pre ℞	1664 (1006.9)	1858 (1424.3)	1786 (1123.3)	***Difference between groups: p<0.001***		
	Post ℞	32 (36.2)	51 (53.6)	1773 (1153.9)	***-1620 (-2422; -817) p<0.001***	***-1794 (-2597; -992) p<0.001***	174 (-601; 950) p=0.65
	Change	*-1633*	*-1807*	*-13.0*			
5βTHF μg/day	Pre ℞	1724 (511.5)	1670 (649.2)	1793 (508.3)	Difference between groups: p=0.96		
	Post ℞	1718 (521.3)	1683 (506.9)	1742 (615.9)			
	Change	*-6*	*+14*	*-52*			

Table 6-1 There was a significant decrease in 5αTHF, and a trend towards a decrease in total urinary glucocorticoids (αTHF+βTHF+THE) following 5αR inhibition. Dutasteride and finasteride did not differ from each other. Data are mean (SD). Change from baseline compared between groups by one-way ANOVA, with LSD post-hoc tests where significant. N, number; GC, glucocorticoids; ℞, treatment; F, cortisol; THF, tetrahydrocortisol; THE, tetrahydrocortisone.

		Dutasteride	Finasteride	Tamsulosin	**Δ Dutasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Finasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Dutasteride vs. Δ Finasteride** Mean (95% CI)
	N	*16*	*16*	*14*			
Total Androgens µg/day	Pre ℞	2623 (1029.9)	3178 (2168.8)	2977 (1731.0)	***Difference between groups: p=0.03***		
	Post ℞	2582 (1388.3)	2107 (1263.5)	3065 (1649.5)	-130 (-1085; 825) p=0.79	***-1159 (-2114; -204) p=0.02***	***1029 (107; 1952) p=0.03***
	Change	*-41*	*-1071*	*+89*			
Aetio (β) µg/day	Pre ℞	817 (403.4)	805 (604.5)	861 (484.6)	***Difference between groups: p<0.001†***		
	Post ℞	2461 (1350.5)	1710 (1002.9)	1029 (658.8)	***p<0.001***	***p=0.01***	p=0.28
	Change	*+1643*	*+905*	*+168*			
Andro (α) µg/day	Pre ℞	1806 (699.2)	2373 (1737.1)	2116 (1297.1)	***Difference between groups: p<0.001***		
	Post ℞	121 (73.0)	397 (310.0)	2036 (1258.5)	***-1605 (-2456;-754) p<0.001***	***-1896 (-2747;-1045) p<0.001***	291 (-1113; 531) p=0.48
	Change	*-1684*	*-1975*	*-79*			

Table 6-2 Finasteride decreased total androgen metabolites after three months of treatment. Both dutasteride and finasteride caused a significant decrease in 5α-reduced, and increase in 5β-reduced androgen metabolites. Data are mean (SD). Change from baseline compared between groups by one-way ANOVA, with LSD post-hoc tests where significant. N, number; ℞, treatment; Aetio, Aetiocholanolone; Andro, androsterone.

			Dutasteride	Finasteride	Tamsulosin	**Δ D vs. Δ T** Mean (95% CI)	**Δ F vs. Δ T** Mean (95% CI)	**Δ D vs. Δ F** Mean (95% CI)
		N	*16*	*16*	*14*			
Glucocorticoids	**βTHF/ αTHF**	Pre ℞	1.39 (0.78)	1.21 (0.73)	1.30 (0.67)	***Difference between groups: p<0.001†***		
		Post ℞	96.20 (66.72)	55.69 (33.36)	1.37 (0.90)	***p<0.001***	***p<0.001***	p=0.65
		Change	*+94.81*	*+54.48*	*+0.07*			
	F/ αTHF	Pre ℞	0.10 (0.10)	0.10 (0.05)	0.12 (0.11)	***Difference between groups: p<0.001†***		
		Post ℞	6.12 (3.68)	4.22 (2.33)	0.13 (0.12)	***p<0.001***	***p<0.001***	p=0.88
		Change	*+6.01*	*+4.11*	*+0.01*			
	F/ βTHF	Pre ℞	0.08 (0.05)	0.09 (0.04)	0.09 (0.05)	Difference between groups: p=0.67		
		Post ℞	0.08 (0.04)	0.08 (0.04)	0.09 (0.04)			
		Change	*+0*	*-0.01*	*0*			
Androgens	**Aetio (β) / Andro (α)**	Pre ℞	0.45 (0.18)	0.36 (0.20)	0.43 (0.14)	***Difference between groups: p<0.001†***		
		Post ℞	21.82 (9.73)	8.26 (15.55)	0.85 (1.43)	***p<0.001***	***p=0.01***	***p=0.01***
		Change	*+21.37*	*+7.89*	*+0.43*			

Table 6-3 Indices of pathways of A-ring reduction of glucocorticoids and androgens by 5αR decreased relative to 5βR following three months of either dutasteride (D) or finasteride (F). Data are mean (SD). Change from baseline compared between groups by one-way ANOVA (F/βTHF), or †Kruskal Wallis test with pairwise comparisons. T, tamsulosin; N, number; THF, tetrahydrocortisol; ℞, treatment; F, cortisol; Aetio, aetiocholanolone; Andro; androsterone.

	Dutasteride	Finasteride	Tamsulosin	**Δ Dutasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Finasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Dutasteride vs. Δ Finasteride** Mean (95% CI)
N	*16*	*16*	*14*			
11β HSD activity measured as ratio of (βTHF + aTHF) / THE						
Pre ℞	0.63 (0.27)	0.67 (0.38)	0.66 (0.23)	***Difference between groups: p=0.001***		
Post ℞	0.35 (0.10)	0.36 (0.16)	0.65 (0.26)	***-0.28 (-0.44; -0.11) p=0.001***	***-0.30 (-0.47; 0.14) p=0.001***	0.02 (-0.14; 0.18) p=0.77
Change	*-0.29*	*-0.31*	*-0.01*			
11β HSD 2 activity measured as ratio of F/E						
Pre ℞	1.18 (0.35)	1.50 (0.62)	1.37 (0.31)			
Post ℞	1.37 (0.51)	1.30 (0.26)	1.34 (0.23)	p=0.15		
Change	*+0.19*	*-0.19*	*-0.03*			

Table 6-4 Urinary glucocorticoid metabolite ratios reflecting activity of 11β-hydroxysteroid dehydrogenase (HSD) enzymes, showing a significant decrease in the index of global 11βHSD activity ((βTHF + αTHF) / THE) following 5αR inhibition, but no change in the index of 11βHSD2 (F/E) activity. Data are mean (SD). Change from baseline compared between groups by one-way ANOVA and LSD posthoc tests where significant. N, number; THF, tetrahydrocortisol; THE, tetrahydrocortisone; ℞, treatment; F, cortisol; E, cortisone.

6.3.2 Effects of 5αR inhibition on circulating concentrations of adrenal steroids

Androstenedione concentrations in serum increased from baseline with dutasteride, compared to both finasteride and tamsulosin. There were no significant differences between treatment groups in DHEAS or basal cortisol concentrations. Results are summarised in Table 6-5.

The pattern of salivary cortisol through the day also did not differ between treatment groups, as seen in Figure 6.1. CBG and calculated free plasma cortisol concentrations also did not change, as summarised in Table 6-6.

		Dutasteride	Finasteride	Tamsulosin	**Δ Dutasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Finasteride vs. Δ Tamsulosin** Mean (95% CI)	**Δ Dutasteride vs. Δ Finasteride** Mean (95% CI)
	N	*16*	*16*	*14*			
AND nmol/L	Pre ℞	3.3 (1.07)	2.9 (1.07)	3.4 (1.78)	***Difference between groups: p=0.002***		
	Post ℞	5.0 (1.72)	3.5 (1.25)	3.9 (2.02)	***1.3 (0.5; 2.0) p=0.002***	0.0 (-0.8; 0.8) p=0.93	***1.2 (0.5; 2.0) p=0.002***
	Change	*+ 1.7*	*+ 0.5*	*+ 0.5*			
DHEAS μmol/L	Pre ℞	0.9 (0.38)	0.8 (0.54)	0.7 (0.43)	Difference between groups: p=0.20		
	Post ℞	0.8 (0.33)	0.7 (0.47)	0.8 (0.57)			
	Change	*- 0.1*	*- 0.1*	*+ 0.1*			
Cortisol nmol/L	Pre ℞	788.5 (236.68)	768.7 (203.49)	817.9 (201.33)	Difference between groups: p=0.88		
	Post ℞	692.1 (186.20)	688.8 (172.89)	757.3 (157.28)			
	Change	*- 96.4*	*- 79.9*	*- 60.6*			

Table 6-5 There was an increase in serum androstenedione (AND) with dutasteride treatment, but no change in serum dehydroepiandrosterone sulphate (DHEAS) or plasma cortisol. Samples are all fasting morning samples. Data are mean (SD). Change from baseline compared between groups by one-way ANOVA, followed by LSD post-hoc tests where significant. N, number; ℞, treatment.

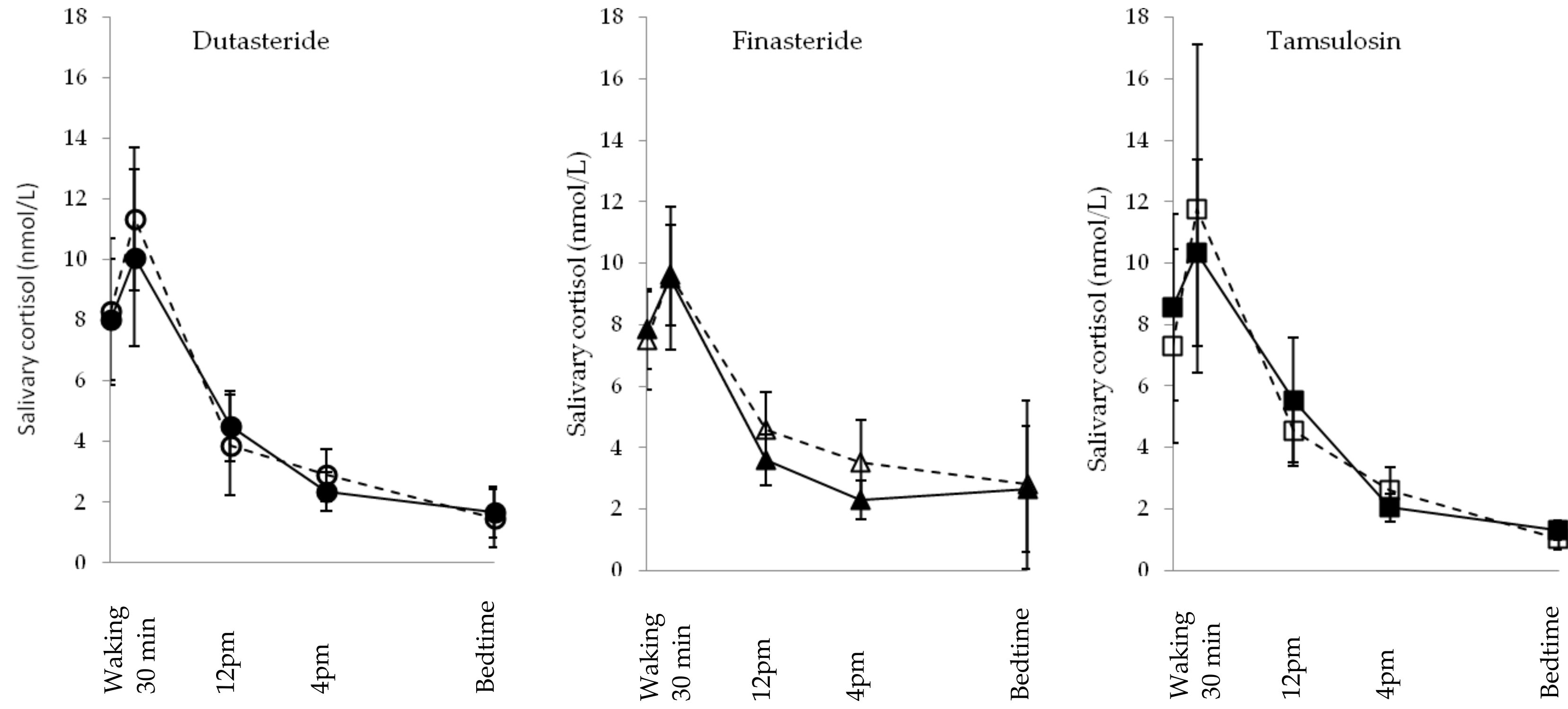

Figure 6.1 Salivary cortisol levels through the day before (open shapes) and after (filled shapes) three months of dutasteride, finasteride or tamsulosin; levels and pattern through the day did not differ in response to treatment. Samples collected at waking, 30 minutes after waking ("30 min"), 12pm, 4pm, and bedtime. Data points are mean; error bars indicate 95% confidence intervals.

		Dutasteride	Finasteride	Tamsulosin	**Difference between groups**
	N	*16*	*16*	*14*	
CBG nmol/L	Pre ℞	988 (172.2)	936 (126.0)	959 (127.4)	p=0.75†
	Post ℞	966 (168.2)	905 (211.6)	929 (156.5)	
	Change	*-22*	*-31*	*-31*	
Plasma free cortisol nmol/L	Pre ℞	60.1 (23.19)	65.9 (33.18)	69.3 (24.47)	p=0.41
	Post ℞	49.4 (23.26)	57.7 (33.74)	65.9 (35.58)	
	Change	*-10.7*	*-8.2*	*-3.4*	

Table 6-6 Plasma corticosteroid binding globulin (CBG) and plasma free cortisol (calculated by Coolens' equation, details in text) did not differ following three months of dutasteride, finasteride or tamsulosin. Data are mean (SD) and change from baseline compared between groups by †Kruskal Wallis test for CBG, and by one-way ANOVA for free cortisol. N, number; ℞, treatment.

6.3.3 Effects of 5αR inhibition on HPA axis responses to suppression and stimulation

Plasma dexamethasone and principal dexamethasone metabolite concentrations were quantified to ascertain whether drug treatment influenced dexamethasone metabolism, prior to interpretation of dexamethasone suppression testing. Changes from baseline did not differ between treatment groups, as shown in Table 6-7.

		Dutasteride	Finasteride	Tamsulosin	**Difference between groups**
	N	*16*	*15*	*14*	
Dex ng/mL	Pre ℞	1.1 (0.60)	0.8 (0.23)	1.1 (0.30)	p=0.39
	Post ℞	0.9 (0.27)	0.9 (0.37)	1.0 (0.38)	
	Change	*-0.2*	*+0.1*	*-0.1*	
6-OH Dex ng/mL	Pre ℞	1.3 (0.62)	1.0 (0.34)	1.1 (0.37)	p=0.21
	Post ℞	1.0 (0.31)	1.1 (0.55)	1.0 (0.36)	
	Change	*-0.3*	*+0.1*	*-0.1*	
11-DH Dex ng/mL	Pre ℞	0.3 (0.16)	0.2 (0.09)	0.2 (0.17)	p=0.17
	Post ℞	0.2 (0.12)	0.3 (0.31)	0.2 (0.13)	
	Change	*-0.0*	*+0.1*	*0*	

Table 6-7 Dexamethasone metabolism was unaltered by drug treatment. Plasma dexamethasone (Dex), 6-hydroxy dexamethasone (6-OH Dex) and 11-dehydroxy dexamethasone (11-DH Dex) concentrations prior to and following three months of dutasteride, finasteride or tamsulosin. N, number; ℞, treatment.

Dexamethasone suppression achieved ~ 45 – 48% suppression from morning cortisol concentrations. In response to dexamethasone suppression there was wide inter-individual variation in suppressed plasma cortisol concentrations and no statistically significant differences were seen, as summarised in Table 6-8. Expressing this data as a percentage of morning plasma cortisol concentrations (fasting samples without overnight dexamethasone suppression) also did not show any difference (data not shown).

Peak cortisol concentrations following Synacthen (post-dexamethasone suppression test) were just in excess of values obtained for unstimulated morning cortisol concentrations, and area under the curve during short Synacthen test did not differ between groups, seen in Table 6-8. The pattern of response to Synacthen is demonstrated in Figure 6.2.

Time to reach peak cortisol level following Synacthen administration was later following 5αR inhibition. As seen in Figure 6.3, most volunteers in the tamsulosin group reached peak plasma cortisol at the same time-point after three months of treatment. In contrast, those on 5αR inhibitors had a greater proportion of subjects who took longer to reach peak cortisol concentration. On Sign Test, the overall change between groups was $p<0.001$, with change from baseline by group: dutasteride $p=0.02$; finasteride $p=0.13$; tamsulosin $p=0.25$.

	Dutasteride	Finasteride	Tamsulosin	**Difference between groups**
N	*16*	*15*	*14*	
Cortisol following low dose dexamethasone suppression test				
Pre ℞	356.6 (238.79)	383.6 (190.33)	368.7 (258.73)	p=0.43
Post ℞	326.2 (260.94)	257.9 (154.10)	366.4 (205.00)	
Change	*- 30.5*	*- 125.8*	*- 2.2*	
Peak cortisol during low dose short Synacthen test				
Pre ℞	868.1 (156.03)	889.9 (232.62)	852.4 (190.68)	p=0.25
Post ℞	842.4 (160.00)	783.1 (110.75)	849.5 (204.47)	
Change	*- 25.7*	*- 106.8*	*- 2.9*	
Area under the curve during short Synacthen test				
Pre ℞	42197 (9095.4)	42552 (10098.4)	42301 (10609.7)	p=0.52
Post ℞	41840 (9048.0)	39906 (9874.9)	412623 (10564.0)	
Change	*- 357*	*- 2646*	*- 1039*	

Table 6-8 Circulating plasma cortisol concentrations (nmol/L) were not significantly different following dexamethasone suppression or Synacthen stimulation following three months of either dutasteride, finasteride or tamsulosin. Area under the curve (nM.minute) of cortisol concentrations during short Synacthen test also did not differ between treatment groups. Data are mean (SD). Change from baseline compared between groups by one-way ANOVA. N, number; ℞, treatment.

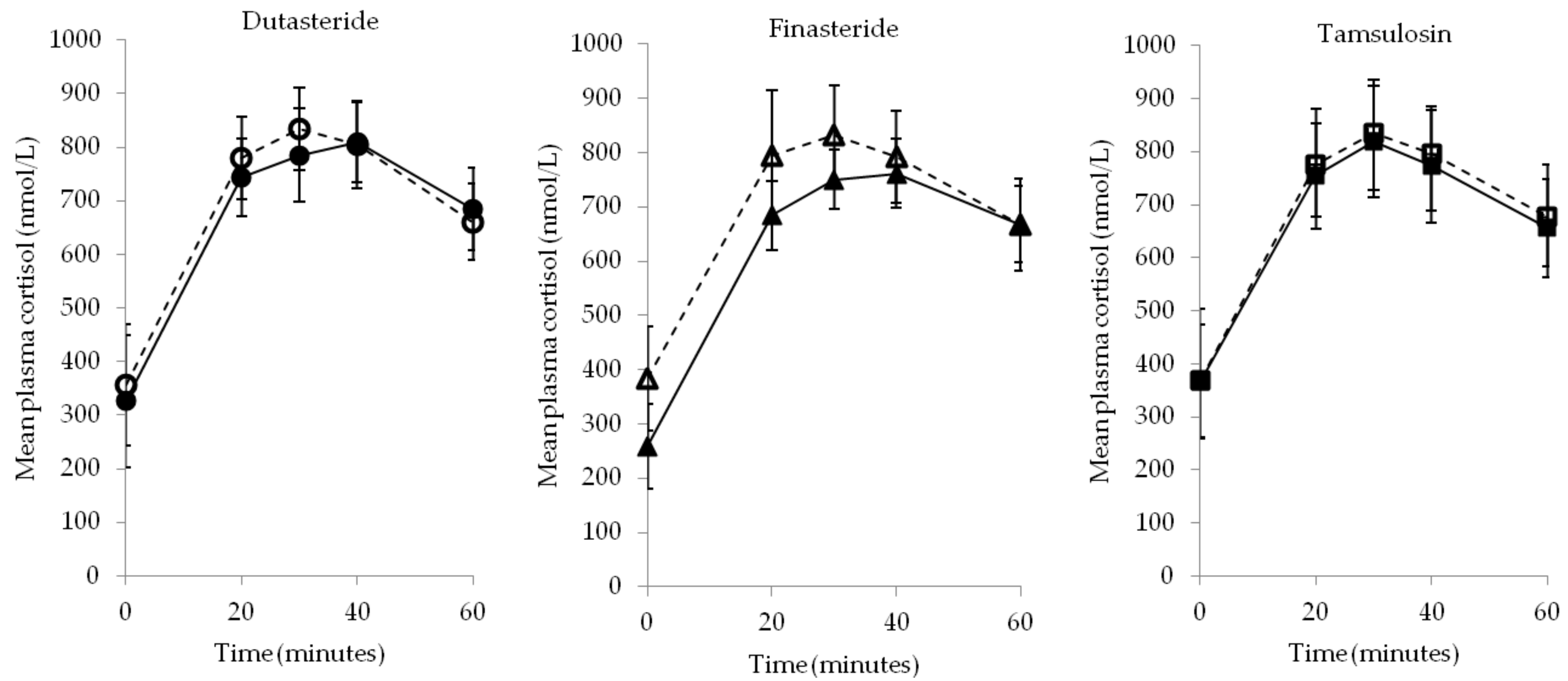

Figure 6.2 Peak cortisol concentrations (p=0.25) and area under the curve (p=0.52) during low-dose (1 µg) short Synacthen test did not differ between groups. Time 0 minutes is the (dexamethasone suppressed) pre-Synacthen value, with Synacthen (IV, 1 µg) administered immediately after. Plasma cortisol was then measured at 20, 30, 40 and 60 minutes post-dose. Open shapes with interrupted lines are before (study day C1) and filled shapes with solid lines are after (study day C2) three months of dutasteride, finasteride or tamsulosin. Data points are mean; error bars indicate 95% confidence intervals; change from baseline was compared between groups by one-way ANOVA.

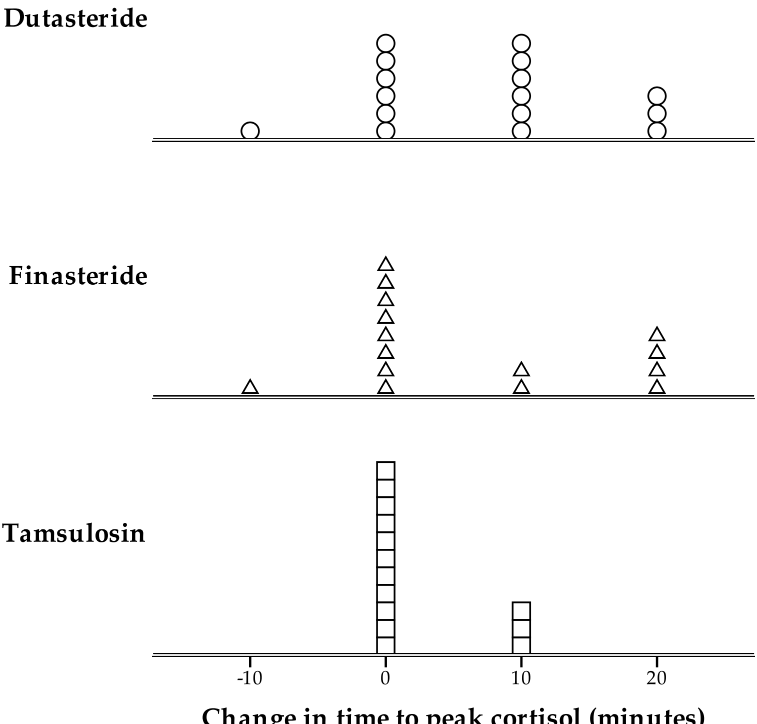

Figure 6.3 The time (minutes) to reach peak plasma cortisol following administration of Synacthen (1 μg, IV) was significantly later (p=0.02 on Sign test) in the dutasteride group. Data represent the change in time (post-treatment – pre-treatment), and each symbol represents a single volunteer.

6.4 Discussion

This chapter has presented investigation of the effects of 5αR inhibition on the HPA axis, studied as a secondary endpoint in a double-blind randomised controlled study, with 46 men. Both dual isozyme inhibition with dutasteride and 5αR2 inhibition with finasteride decreased 5αR metabolism of cortisol and the HPA axis was able to compensate for this decrease in cortisol clearance to maintain normal cortisol concentrations in plasma and saliva. No changes in response to suppression of the HPA axis by dexamethasone were seen, however subtle changes in response to stimulation were indicative of minor adrenal insufficiency. Serum androstenedione was increased with dutasteride only, and androgen metabolism by 5βR was increased as a compensatory response to 5αR inhibition with both dutasteride and finasteride.

As anticipated, 5α-reduction of glucocorticoids was significantly inhibited by dutasteride and finasteride. It was possible that other metabolic routes may have compensated for inhibition of 5αR; however the products of metabolism of glucocorticoids by 5βR were unaltered, in line with a lack of change in cortisol as its substrate. In contrast, the conventional index of 11βHSD activity was decreased, and in the absence of a change in 11βHSD2 activity this implies a decrease in 11βHSD1 activity and is indicative of impaired cortisol regeneration. While this is plausible as a means to

compensate for impaired clearance, this index must be interpreted with caution. Given the equation for calculation of 11βHSD activity is highly dependent on 5αTHF, this may be influenced purely as a result of the decrease in 5αR activity, as has been suggested when a similar change was seen with finasteride treatment (Christakoudi et al., 2003). Indeed, if 11βHSD activity is inferred from βTHF/THE then there was no difference between groups (data not shown). However 11βHSD1 activity may indeed decrease with 5αR inhibition, perhaps as a compensatory protective down-regulation to attenuate the proposed increase in local glucocorticoid amounts upon 5αR inhibition (Pelletier et al., 2007). While 5αR knockout mice had no change in liver 11βHSD1 transcript or protein (unpublished), there may be species differences between rodents and humans. End product inhibition due to cortisol accumulation is unlikely to be a contributing factor as 11βHSD1 in human liver microsomes is not thought subject to this (up to 20 x physiological concentrations) (Diederich et al., 2000).

Overall, accompanying impaired metabolism by 5αR, there was a trend to suppress total amounts of urinary glucocorticoids, indicative of a decrease in daily production rates, with both 5αR inhibitors. This finding supports the hypothesis that compensation by the HPA axis to down-regulate synthesis occurs in response to impaired clearance. The degree of decrease in urinary 5αTHF and total glucocorticoids was not significantly different between

dutasteride and finasteride, and indeed inhibition of 5αR2 alone appeared to achieve ~97% suppression of 5αTHF. Finasteride *in vivo* is only thought to effectively inhibit 5αR2, not 5αR1, with K_m values of <0.1 nM and 52 respectively (Aggarwal et al., 2010), therefore these findings are believed reflective of 5αR2 inhibition. This suggests that 5αR2 makes a highly significant contribution to cortisol metabolism. Presumably this is reflective of 5αR2 in the liver which is the primary site of cortisol metabolism by 5α-reductase (Gold, 1961), although enzyme expressed in other peripheral sites may also be involved. Interestingly, in 2 patients with 5αR2 deficiency a similar decrease in cortisol 5α urinary metabolites has also been described, though a quantitative description was not published (Fisher et al., 1978).

As with 5αR1-/- mice under basal conditions, this suppression of glucocorticoid synthesis allowed the HPA axis to compensate for decreased 5αR-mediated clearance and to maintain plasma cortisol concentrations within the normal range. Measures of basal, unstressed cortisol, obtained by sampling saliva across the day and specifically at the evening nadir, were unchanged. Cortisol circulates in blood bound to albumin and corticosteroid binding globulin (CBG) and measurements in saliva, a filtered fluid not allowing passage of proteins, allows for assessment of the free bioavailable portion. This was thought especially important at study planning, since with decreased hepatic clearance of cortisol, it was possible that hepatic CBG

synthesis could be decreased (Cole et al., 1999), making assessment of total circulating cortisol potentially misleading; circulating free cortisol may have increased, without affecting the total amount. Salivary cortisol levels did not differ between groups, indicating that (at least with this duration of treatment) CBG was unlikely to have been affected (Vining et al., 1983), and this was indeed corroborated by measurement of CBG and calculation of plasma free cortisol.

Salivary cortisol also provides a complementary non-invasive measure of the diurnal profile of free cortisol (Kirschbaum and Hellhammer, 1994). The pattern of salivary cortisol did not differ between groups in this study, and in particular there was no 'flattening' of the saliva curve, as has been reported in obesity, suggesting morning cortisol concentrations were not lower and evening cortisol concentrations were not disproportionately higher (Rosmond et al., 1998) following 5αR inhibition, despite metabolic changes in the group receiving dutasteride.

Differences in morning plasma cortisol concentrations were not seen between groups. In conjunction with this finding, there was also no difference in the so-called "cortisol awakening response" (Pruessner et al., 1997), measured as the increment rise between waking to 30 minutes after waking, a measure thought to reflect adrenal stimulation in which an increase has been

associated with the metabolic syndrome by some (Bengtsson et al., 2010) but not others (DeSantis et al., 2011). Notably the main difference seen here is in a lower morning cortisol level compared to healthy controls, and then a greater proportional rise after waking (Bengtsson et al., 2010).

Dynamic testing of the HPA axis did not reveal marked differences between treatment groups, indicating that 5αR inhibition did not impair response to stimulation of the axis to any great degree. A short Synacthen (1 µg) test was executed at doses chosen to elicit EC_{50} responses, rather than maximal responses which would result from conventional doses (250 µg) (Reynolds et al., 2001, Daidoh et al., 1995, Dickstein et al., 1991). Despite only using a low dose, it can be seen that peak cortisol levels were ~850 nmol/L, which is considerably higher than peak concentrations of ~400 nmol/L reported elsewhere (Reynolds et al., 2001, Dickstein et al., 1991). However immunoassays differ considerably (Cohen et al., 2006), and a clearer picture is provided when interpreting these findings in conjunction with the basal morning cortisol levels; while cortisol levels following Synacthen look 'high', it can be seen that the basal morning cortisol levels are similar, supporting that the aim of the test in stimulating sub-maximal response was likely achieved. In addition, comparison to the dexamethasone suppressed plasma cortisol concentrations shows an approximately two-fold increase upon Synacthen stimulation, in keeping with others (Reynolds et al., 2001). The

time to peak cortisol during the short Synacthen test was delayed in those on dutasteride, with finasteride following a similar pattern, though this was not significant. This is reflective a delayed adrenal response to stimulation (Reynolds et al., 2001), and supports the hypothesis of a down-regulated HPA axis, though not to a great degree.

The preservation of a relatively normal response following 5αR inhibition differed from the response seen in 5αR -/- mice, where the ability to mount a corticosterone response to restraint stress was substantially attenuated (Livingstone et al., 2010). The lack of difference seen in the present study may simply reflect an under-powered study, as assessment of the HPA axis was a secondary endpoint. However in the mice disruption of 5αR1 slowed clearance by greater than 50% (unpublished), whereas in humans the effect of even dual 5αR inhibition only attenuated glucocorticoid production by ~20%. Therefore mice may be more dependent on this metabolic pathway, and in humans it may be necessary to inhibit either 5β-reduction, both A-ring reductases or 11βHSD2 to induce adrenal insufficiency. In addition the treatment duration of 3 months may not have been sufficient to clearly demonstrate such a phenotype. The responses to dynamic testing in Zucker rats treated with finasteride for 1 month (where inhibition of 5αR1 would be less pronounced than in 5αR -/- mice) showed no change in response to

restraint, (unpublished), and this could perhaps be considered a more comparable model to the human study.

With expression of 5αR in the brain (Table 1-1, Introduction) there is potential for 5αR inhibitors to have a central effect on the HPA axis, however there was no evidence for this in the present study. Had glucocorticoids accumulated centrally then cortisol concentrations would be expected to fall, however this was not seen. If GR levels had exhibited some degree of autoregulation (Rupprecht et al., 1991) to adjust to increased glucocorticoid concentrations, this would have manifest in the dexamethasone suppression test with decreased suppression of plasma cortisol, and once again there was no evidence for this.

In contrast to normal circulating cortisol concentrations, serum androstenedione was increased with dutasteride only, likely a reflection of substrate accumulation following 5αR inhibition, which was more marked with the more efficacious drug. Androstenedione is a known substrate for 5αR in both normal and BPH-affected human prostate (Weisser and Krieg, 1997), with a K_m value of 1.7 μM compared to 3.6 μM for testosterone (Andersson and Russell, 1990). Finasteride inhibits equally well the 5α-reduction of both androstenedione and testosterone (Weisser and Krieg, 1998); the activity of dutasteride in inhibiting androstenedione is not

described, though is likely to be equal if not more effective than finasteride. The post-treatment androstenedione concentrations in the dutasteride group were just slightly in excess of the reference range reported by some (Kushnir et al., 2010a), and well within those used in the NHS laboratories in Edinburgh (NHSLothian, 2011-2013). This finding is also in agreement with previous studies; one month of finasteride does not increase androstenedione (Rittmaster et al., 1994), and concentrations in two patients with 5αR2 deficiency were surprisingly slightly lower than unaffected men (Fisher et al., 1978). In contrast, as seen here, with 3-6 months of dutasteride treatment (co-administered with testosterone) there is a significant increase in androstenedione, which was more marked than seen with testosterone administration alone (Page et al., 2011a).

Dutasteride and finasteride both exhibited similar effects on urinary androgen metabolites, which are largely a reflection of adrenal androgen synthesis (Weykamp et al., 1989, Finken et al., 1999) and this corroborates less forward drive to synthesis by the HPA axis. An expected decline in the 5α metabolite androsterone was seen with both dutasteride and finasteride and there was a compensatory increase in androgen metabolism by 5βR. Compensation was unable to compensate fully for those treated with dutasteride, leaving a residual increase in serum androstenedione. An additional contributing factor may be that while cortisol is closely regulated

by the HPA axis (Jacobson, 2005), regulation of androstenedione synthesis is not so tightly controlled (Takizawa et al., 2010), therefore rather than suppression of its synthesis, increased metabolism by 5βR may be the preferred clearance route. Androstenedione has a lower K_m (4.85 μM) than cortisol (11 μM) for 5βR metabolism (Okuda and Okuda, 1984), and this may explain the increase in 5βR metabolism of androgens but not glucocorticoids. As NADPH is cofactor for both enzymes (Frederiksen and Wilson, 1971, Berseus and Bjorkhem, 1967, Kondo et al., 1994), with a decrease in 5αR activity there could in theory be an increase in cofactor availability for 5βR. This is unlikely however as 5αR is predominantly microsomal (Russell and Wilson, 1994) and 5βR is thought to be predominantly cytosolic, though has been reported in all sub-cellular fractions (Iyer et al., 1990).

While total androgen urinary metabolites were decreased with finasteride, the decrease in 5αR metabolism was largely balanced by the increase in 5βR metabolism with dutasteride. This presumably reflects less need for 5βR compensation following finasteride treatment, however may also reflect a degree of 5βR inhibition with finasteride (Drury et al., 2009).

The increase in serum androstenedione may reflect an increase in adrenal steroidogenesis, though notably an increase in DHEAS (which is not a known substrate of 5αR) was not seen. While modulated by the HPA axis,

adrenal androgens do not always show the same pattern of secretion (and therefore probably regulation) as cortisol (McKenna and Cunningham, 1991, Bornstein and Chrousos, 1999). Other effectors of adrenal androgen synthesis are, however, less well understood. Insulin *in vitro* (Kramer et al., 1990) and free fatty acids *in vivo* (Mai et al., 2006) have been reported to selectively stimulate adrenocortical androgen rather than glucocorticoid release; making it possible that the hyperinsulinaemia seen in the dutasteride group (Chapter 5.4.6.1) may by contributing to the increased androstenedione concentrations. An increase in sensitivity to ACTH in androgen synthesising cells has been reported in PCOS patients (Azziz et al., 1998), though in contrast in men cortisol may be more sensitive than androstenedione to Synacthen infusion (Parker et al., 1996). Overall differing sensitivity of adrenocortical cells has not been studied in detail in men and at present it is unknown whether this played any role in the findings seen.

While principal feedback to the HPA axis is from glucocorticoids (Jacobson, 2005), androgens and oestrogens may also play a role; an important consideration given that following 5αR inhibition circulating androstenedione was increased, DHT decreased (Chapter 5.4.5.1), and there was a trend towards an increase in oestradiol (Chapter 5.4.13). Normally testosterone (Viau and Meaney, 1996) and DHT (Lund et al., 2006) both modulate the HPA axis, with a net effect to decrease circulating

glucocorticoids. Effects of DHT are also thought to be mediated by its (ERβ ligand) metabolite, 3β-diol, which also has an inhibitory effect (Handa et al., 2011), and in theory would also decrease following 5αR inhibition. Androstenedione is not thought to play an important role in HPA feedback in men. Therefore the usual inhibitory effect exerted by DHT would be attenuated in the context of 5αR inhibition, and in turn increase adrenal steroidogenesis; the opposite of what was observed, and likely a reflection of the greater role of cortisol in HPA feedback than DHT. Similarly oestradiol is thought to cause a hyper-responsive HPA axis (Kirschbaum et al., 1996), therefore in this setting the trend towards an increase in oestradiol is more likely to be an effect of 5αR inhibition, rather than a cause of HPA axis dysfunction.

In conclusion, this chapter has described the effects of 5αR inhibition on the HPA axis in men. There was a significant decrease in cortisol metabolism by 5αR with both dutasteride and finasteride. There were subtle signs of adrenal insufficiency following Synacthen stimulation, however there was preservation of normal circulating cortisol concentrations and the HPA axis was able to compensate for the effects of 5αR inhibition. A reduction in 5αR metabolism of androgens was compensated for by an increase in 5βR activity following both 5αR inhibitors, however incomplete compensation with dutasteride resulted in a residual increase in circulating androstenedione

concentrations. This study has demonstrated the first reported detailed measure in a human population of HPA axis function with both finasteride and dutasteride, and demonstrates differential adaptation of the HPA axis to glucocorticoids and androgens following inhibition of 5αR.

Chapter 7: Conclusions

The aim of this thesis was to address the paucity of knowledge regarding the metabolic effects of 5αR inhibition in man. The central hypothesis was that, due to expression in metabolic tissues where androgen and glucocorticoid homeostasis are affected, inhibition of 5αR1 with dutasteride would be detrimental to metabolic health. A double-blind randomised controlled study was conducted, with 46 men studied prior to and following three months of treatment with dutasteride (dual inhibitor of 5αR1 and 5αR2), finasteride (5αR2 inhibitor) or tamsulosin (α blocker, control group). 5αR inhibition with dutasteride, but not finasteride, resulted in a significant decrease in peripheral insulin sensitivity and increase in body fat. In addition, there was evidence of subtle dysregulation of the HPA axis with both 5αR inhibitors.

Excess tissue glucocorticoid is associated with features of the metabolic syndrome (Walker, 2001). One of the rate-limiting steps in glucocorticoid degradation is catalysed by 5αR (Shamim et al., 2000, Baudrand et al., 2011), therefore in theory, inhibition of 5αR1 may lead to an accumulation of cortisol in metabolic tissues. The two best characterised isozymes of 5αR differ in their tissue distribution: 5αR2 is predominantly expressed in the reproductive tract and liver (Russell and Wilson, 1994, Shirakawa et al., 2004), while 5αR1 is expressed in metabolic tissues including liver and adipose (Russell and Wilson, 1994, Wake et al., 2007). Well known for catalysing the conversion of the androgen testosterone to the more potent

dihydrotestosterone, 5αRs are inhibited in the treatment of androgen target tissue disorders, most notably benign prostatic hyperplasia (Aggarwal et al., 2010). However, 5αR inhibition may also affect other substrates of 5αR, including glucocorticoids.

Research in animal models has demonstrated that mice with genetic disruption of 5αR1 develop glucose intolerance and fatty liver, particularly when metabolically challenged with a high fat diet and female mice are more susceptible (Livingstone et al., 2008, Livingstone et al., 2009a). These results were recapitulated with pharmacological inhibition of 5αR1 and 5αR2 in obese Zucker rats (Livingstone et al., 2009a) and these findings are not androgen dependent, in that they persist in castrated animals. In humans, previous studies of metabolic effects of 5αR inhibition have been limited to measures of fasting glucose (Amory et al., 2007).

This thesis has described the implementation of a double-blind randomised controlled clinical study, together with validation and application of novel supporting assays. Three months of treatment with dutasteride significantly impaired insulin sensitivity, decreasing glucose disposal by 5.7 μmol/kgFFM/min (~14%) during high-dose insulin infusion. This was suggestive of a peripheral insulin resistance, and together with changes in

other surrogates of peripheral insulin sensitivity, skeletal muscle and adipose were implicated as the primary sites of impaired insulin action.

Given the clear differences between dutasteride and both finasteride and control, steps to determine the mechanism were a key focus of the work presented; though as yet an exact mechanism remains elusive. It is implied in study findings that the metabolic effects of dutasteride must lie in the inhibition of 5αR1, as similar findings were not seen with finasteride. Circulating DHT and cortisol concentrations did not differ between groups, therefore could not account for this difference. Oestradiol had a trend towards increasing following 5αR inhibition, and rose more so with dutasteride. This remains a possible mediator, as both ERα and ERβ mRNA and protein are expressed in human skeletal muscle (Wiik et al., 2009). However, while oestrogens are associated with metabolic disease (Vikan et al., 2010, Phillips et al., 2003), the mechanism behind which they may impair skeletal muscle insulin sensitivity in men is not understood. Oestrogen effects on glucose homeostasis are thought to be mediated predominantly via ERα (Foryst-Ludwig and Kintscher, 2010), and if this is case then the probable decrease in the ERβ ligand 3β-diol (a DHT metabolite) with 5αR inhibition would likely not contribute to the phenotype seen. Future development of mass spectrometry based assays may provide a more reliable indicator of oestrogen concentrations, and given previous reports of normal

oestradiol concentrations following a year of treatment (Amory et al., 2007) the possibility remains that this was a transient feature. Inhibition of 5αR3 (Godoy et al., 2011, Uemura et al., 2008) in this study was not specifically studied; however with increasing knowledge about this isozyme future studies may explore this possibility.

Tissue accumulation of glucocorticoids was the underpinning mechanism thought responsible, however while liver is the primary site of glucocorticoid metabolism (Gold, 1961), there was no evidence of a hepatic insulin resistance phenotype with dutasteride. Indeed, the importance of both 5αR1 and 5αR2 in hepatic glucocorticoid metabolism was demonstrated in this study with similar inhibition of 5αTHF seen with both inhibitor drugs. Tracer kinetics indicated hepatic insulin sensitivity was preserved, and there was no evidence of fatty liver on post-treatment ^{1}H spectroscopy. There are of course inherent limitations in human studies, and it may be that fatty liver seen in animal models (Livingstone et al., 2008, Livingstone et al., 2009a) was not apparent with a short duration of treatment, that a post-study only measure of liver fat was not sufficiently sensitive to distinguish groups, or that impairment of hepatic insulin sensitivity is not a feature of human 5αR inhibition. It is also reasonable to consider that the early pathogenesis of the metabolic syndrome may differ between rodents and humans.

Body composition was altered with increased body fat in those on dutasteride only, and a degree of adipose insulin resistance was evident with less suppression of plasma NEFA levels following low-dose insulin infusion. However there was no change in the circulating adipokines or chemokines quantified, nor any alterations in gene transcript abundance to indicate metabolic or inflammatory changes in subcutaneous adipose. The increase in body fat was unable to be localised to a specific abdominal depot on MRI. In future studies, if resources permit and necessary technological advances are achieved, novel imaging techniques such as positron emission tomography (PET) scanning (Ng et al., 2012) may provide valuable non-invasive measures of glucose metabolism, enabling physiological assessment of both subcutaneous and visceral adipose.

Local metabolism of steroids by 5αR1 within muscle, or the deposition of ectopic fat are possible mediators of metabolic effects seen in this study. Muscle biopsies and muscle spectroscopy were not performed in this study; therefore the deposition of fat or alteration in glucose metabolism post dutasteride treatment is unknown. Further ongoing work is in determination of relative expression of 5αR isozymes in metabolic tissues of interest in both rodent and human. However, as has been demonstrated previously (Stuerenburg and Schoser, 1999) 5αR mRNA and protein expression may not translate to detectable activity. In order to further our understanding, the use

of arterio-venous sampling across a muscle bed may give some insight regarding muscle 5αR activity, in particular whether cortisol is metabolised by muscle 5αR. Muscle androgen metabolism could be further investigated with the use of deuterated testosterone tracers (Vierhapper et al., 1997, Wang et al., 2004b). In addition, a further valuable mechanistic investigation may be a cross-sectional clinical study with muscle spectroscopy performed in patients on dutasteride and finasteride compared to healthy controls, in particular looking for evidence of increased triglyceride in muscle, and/or muscle biopsies taken to assess for glycogen synthesis.

Metabolic dysfunction is associated with impairments of the HPA axis (Pasquali et al., 2006), and vice versa (Rosmond et al., 1998). The clear metabolic changes induced by dutasteride treatment, together with expression of 5αR in sites potentially modulating the HPA axis (Thigpen et al., 1993, Russell and Wilson, 1994), may have altered function of the HPA axis. Investigated as a secondary endpoint in this study, there was a clear decrease in 5αR metabolism of both glucocorticoids and androgens, together with evidence of compensation. For glucocorticoids, compensation was by down-regulation of the HPA axis with decreased glucocorticoid production and maintenance of a normal circulating cortisol concentration. Androgens compensated largely by increasing metabolism by 5βR, with a residual increase in serum androstenedione with dutasteride, evidence of substrate

accumulation. Pituitary response to dexamethasone suppression was normal; however there was evidence of a delayed peak cortisol response with adrenal Synacthen stimulation suggesting a degree of adrenal insufficiency. Overall, testing of the HPA axis revealed an ability to compensate for 5αR inhibition maintaining normal circulating cortisol concentrations. This observation naturally lends itself to the question of whether tissue glucocorticoid concentrations were also normalised. It is impossible to definitely establish this in this setting, however given ongoing metabolic abnormalities potentially driven by cortisol accumulation this is suggestive of a continuing elevation in tissue glucocorticoid concentrations. The use of deuterated cortisol tracers (Hughes et al., 2010) may in future provide more detailed information about peripheral tissue-specific glucocorticoid turnover following 5αR inhibition.

The study presented sampled a subset of men, consisting mostly of healthy volunteers. As with all studies, sample groups cannot reflect the entire population and this must be considered before extrapolation of results to individual patients or communities. It could however be hypothesised that with declining β cell function (Ma et al., 2009) and increased body fat (Boden et al., 1993), older men would be more susceptible to metabolic consequences of 5αR inhibition than seen in the present study; rendering these results even more relevant to the 'typical' BPH patient. The probable association of BPH

with the metabolic syndrome (Moul and McVary, 2010, Parsons et al., 2006) and long-term prescription of 5αR inhibitors to patients with additional predispositions to metabolic disease can only make these data even more clinically relevant.

The common usage of these medications also makes the work in this thesis potentially widely clinically applicable. Annually in Britain there are ~500,000 dutasteride, ~2.3 million finasteride, ~4.7 million tamsulosin, and ~70,000 combined dutasteride/ tamsulosin prescriptions dispensed (Wales, 2011) (England, 2011) (Scotland, 2011). In men, finasteride is licensed for use in BPH and androgenic alopecia, and in women it is licensed for treatment of hirsutism. Dutasteride is licensed for use in BPH only, as are the combined dutasteride/ tamsulosin preparations. Given the adverse metabolic consequences of 5αR inhibition with dutasteride presented here, and the lack of evidence to suggest dutasteride is a more effective treatment (Andriole and Kirby, 2003, Nickel et al., 2011), it may be that finasteride should be the preferred drug in BPH. One consideration however is that finasteride is not available in a pre-prepared combination with an alpha blocker, meaning this approach may be less acceptable to patients needing combination treatment.

Despite the demonstrated impairment of insulin sensitivity, there are select clinical scenarios where dutasteride may still be the preferred 5αR inhibitor:

treatment of castration-resistant prostate cancer (CRPC), prostate cancer prevention, and prevention of complications from BPH. Dutasteride is possibly more effective than finasteride in lowering prostatic DHT concentrations (van der Sluis et al., 2012), but perhaps of greater importance the majority of prostate cancers express predominantly 5αR1 and the most commonly utilised prostate cancer cell line, LNCaP, expresses only 5αR1 and not 5αR2 (Negri-Cesi et al., 1998). Conversely, dutasteride may also have deleterious effects and in CRPC has been shown promote androstenedione metabolism to testosterone with its own AR agonist effects (Chang et al., 2011). In addition, glucocorticoids (both endogenous and synthetic) are agonists for a mutated high-affinity cortisol-cortisone responsive AR seen in CRPC (Zhao et al., 2000, Chang et al., 2001). The balance between these factors, and the clinical importance and implications of dutasteride in these conditions are still being established, however if proven beneficial then a decrease in insulin sensitivity would clearly be of diminished relative importance in such patients.

This thesis has focused on the effects of 5αR inhibition in men. Healthy women could not be studied in a similar manner as dutasteride is not licensed for use in women and risks to a male foetus if volunteers conceived during (or soon after) the study would be unacceptable. However, with high expression of 5αR1 in non-genital skin (Eicheler et al., 1995), any proposed

use of dutasteride in hirsutism treatment, for example in PCOS, may be affected by results in this thesis as PCOS itself is associated with insulin resistance (Amato et al., 2006). 5αR isozyme distribution is not thought to differ between men and women, therefore it could be expected that finasteride in women would not induce insulin resistance, though all that is known to date is that fasting glucose levels do not change (Moghetti et al., 2000). The metabolic effects of sex steroids, and changes in sex steroids, are manifest in a gender-specific manner (Wu and von Eckardstein, 2003), and what effect dutasteride may have on sex steroids in women is unknown, though in 5αR -/- mice females were more susceptible to metabolic disease than males (Livingstone et al., 2008).

Dutasteride induces peripheral insulin resistance, and insulin resistance is of key prognostic importance. As demonstrated in a landmark study in a Pima Indian population (Lillioja et al., 1993), insulin resistance (independently of obesity) was a significant risk factor for the development of type 2 diabetes after 5 years of follow-up. In addition, skeletal muscle insulin resistance, the primary feature seen with dutasteride treatment, is thought to be the key antecedent to future diabetes (DeFronzo and Tripathy, 2009). Therefore findings presented of increased insulin resistance with only 3 months of dutasteride therapy naturally beg the question of the potential long-term implications of dutasteride treatment. An approach to this question would be

to conduct a retrospective analysis of the incidence of diabetes in patients taking dutasteride compared to finasteride or tamsulosin; data which (in contrast to detailed measures of insulin sensitivity) would be more readily available in population health databases. In addition, the progression or complications of metabolic disease may be more apparent in patients with diabetes treated with dutasteride. Both of these studies are now underway.

In conclusion, this thesis has presented the first detailed study in humans comparing the metabolic effects of inhibiting 5αR2 with finasteride, to the effects of dual isozyme inhibition with dutasteride. There was a significant and likely clinically relevant decrease in peripheral insulin sensitivity and increase in body fat with dutasteride. In addition, testing of the HPA axis revealed subtle dysregulation with both 5αR inhibitors, though compensation for this was evident. The work presented in this thesis has highlighted a novel determinant of metabolic health with potentially important implications for BPH patients, and results lead now to further exciting avenues of potential research, particularly in exploring mechanisms underpinning the metabolic consequences of dutasteride treatment.

Chapter 8: References

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