An investigation into the biosynthesis and metabolism of adrenal C11-oxy steroids

by

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Declaration

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This dissertation includes 2 original papers published in peer-reviewed journals, 1 paper under review and 1 to be submitted for publications. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

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Summary

This study describes:

- an *in vitro* investigation into the biosynthesis and metabolism of 11β-hydroxyprogesterone (11OHP4) and 21-deoxycortisol (21dF) by steroidogenic enzymes which include the 5α-reductases, 3α-reductases and cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1), as well as the interconversion of the C11-oxy C_{21} steroids by the 11β-hydroxysteroid dehydrogenase isozymes.

  The CYP17A1-catalysed conversion of the C11-oxy C_{21} steroids showed that while 11OHP4 and 11KP4 were readily hydroxylated at C17, negligible lyase activity was detected towards 21dF, 21-deoxycortisone (21dE) and the 5α-reduced C11-oxy C_{21} metabolites, while the 3α,5α-reduced C11-oxy C_{21} metabolites were converted to the 3α,5α-reduced C11-oxy C_{19} steroids. Homology modelling of 11OHP4, 5α-pregnan-11β-ol-3,20-dione (11OH-DHP4), and their keto derivatives, showed that these steroids have similar orientations in the active site of CYP17A1 compared to P4 while the orientation of 5α-pregnan-3α,11β-diol-20-one and 5α-pregnan-3α-ol-11-20-dione was similar to 17α-hydroxypregnenolone.
- the transactivation by the androgen- (AR) and progesterone receptor (PR) isoform A and B in response to the C11-oxy C_{21} steroids, and transactivation by the PR isoforms in response to C11-oxy C_{19} steroids.

  At 100 nM, 11OHP4, 11OH-DHP4 and 21dF exhibited agonist activity towards the AR, PR-A and PR-B, with comparable agonist activity at 10 nM with the exception of 21dF. The 11-keto derivatives exhibited negligible agonist activity towards the AR and PR. The drawbacks of commonly used luciferase reporter promoter assay protocols were highlighted.
- steroid profile analysis of serum and salivary samples of male athletes provided evidence of C11-oxy backdoor pathway activity *in vivo*. Analysis of unconjugated circulating steroids generated comprehensive steroid profiles. No significant differences in steroid levels were detected between the two age groups and the ethnic groups. Fewer steroids were detected in salivary samples with the C11-oxy steroids being the predominant steroids. Inter-individual variation in steroid levels highlighted the clinical benefits of comprehensive steroid profiling for the diagnosis and monitoring of endocrine conditions.

- the development and validation of ultra-performance convergence chromatography tandem mass spectrometry (UPC^2-MS/MS) methods which were used for the detection and quantification of C_{19}, C_{21}, C11-oxy C_{19} and/or C11-oxy C_{21} steroids in cell culture medium, in serum and in salivary samples.

Opsomming

Hierdie studie beskryf:

- 'n ondersoek *in vitro* na die biosintese en metabolisme van 11β-hidroksieprogesteroon (11OHP4) en 21-deoksiekortisol (21dF) deur steroïedogeniese ensieme wat die 5α-reduktases, 3α-reduktases en sitochroom P450 17α-hidroksilase/17,20-liase (CYP17A1) insluit, sowel as die omsetting van die C11-oksie C_{21}-steroïede gekataliseer deur die 11β-hidroksisteroïeddehidrogenase-isosieme.

  Die CYP17A1-gekataliseerde omsetting van die C11-oksie C_{21}-steroïede het getoon dat alhoewel 11OHP4 en 11KP4 geredelik op posisie C17 gehidroksileer word, die verdere omsetting van 21dF, 21-deoksiekortisoon (21dE) en die 5α-gereduseerde C11-oksie C_{21}-metaboliete, gekataliseer deur die liase aktiwiteit, minimaal was. Slegs die 3α,5α-reduseerde C11-oksie C_{21}-metaboliete is omgesit na 3α,5α-reduseerde C11-oksie C_{19}-steroïede. Homologie modelering van 11OHP4, 5α-pregnan-11β-ol-3,20-dioon (11OH-DHP4), en hul ketoderivate het getoon dat hierdie steroïede en P4 'n soortgelyke oriëntasie in die aktiewe setel van CYP17A1 het, terwyl die oriëntasie van 5α-pregnan-3α,11β-diol-20-oon en 5α-pregnan-3α-ol-11-20-dioon soortgelyk is aan 17α-hidroksiepregnenoloon.
- die transaktivering van die androgeen- (AR) en progesteroonreseptor (PR) isovorme A en B deur die C11-oksie C_{21}-steroïede, sowel as die PR-isovorme deur die C11-oksie C_{19}-steroïede. By 'n konsentrasie van 100 nM het 11OHP4, 11OH-DHP4 en 21dF agonisaktiwiteit teenoor die AR, PR-A en PR-B getoon, agonisaktiwiteit by 10 nM was vergelykbaar, met die uitsondering van 21dF, wat geen aktiwiteit getoon het nie. Die 11-ketometaboliete het swak tot geen agonis aktiwiteit teenoor die AR- en die PR-isovorme getoon. Die tekortkominge van die eksperimentele protokol vir reseptortransaktivering in HEK-293 en soortgelyke selmodelle is bespreek.
- die steroïedprofielanalise van serum- en spoegmonsters van mansatlete. Die C11-oksie C_{21}-steroïede en die C11-oksie C_{19}-steroïede is *in vivo* in hul spesifieke metaboliese paaie geïdentifiseer. 'n Omvattende steroïedprofiel is geskep deur die analise van die ongekonjugeerde sirkulerende steroïede, en geen beduidende verskille in steroïedvlakke tussen die twee ouderdomsgroepe of etniese groepe is gevind nie. Minder steroïede was in spoeg monsters gekwantifiseer en steroïede met 'n C11-oksiegroep het meer geredelik voorkom. Inter-

individuele variasie in steroïedvlakke beklemtoon die kliniese belang van omvattende steroïedprofiele vir die diagnose en monitering van endokriene kondisies.

- die ontwikkeling en validering van UPC^2-MS/MS metodes wat toegepas is vir die deteksie en kwantifisering van C_{19}, C_{21}, C11-oksie C_{19} en/of C11-oksie C_{21}-steroïede in selkultuurmedium, serum en in spoegmonsters.

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Vir Den – jy is my alles.

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Contents

Abbreviations

General

17OHD	17α-hydroxylase/17,20-lyase deficient
21CAH	21-hydroxylase deficiency congenital adrenal hyperplasia
21OHD	21-hydroxylase deficiency
ABP	athlete biological passport
ACTH	adrenocorticotropin-releasing hormone
ADT	androgen deprivation therapy
AngII	angiotensin II
$_{app}K_m$	apparent K_m
AR	androgen receptor
ARPI	androgen receptor pathway inhibition
BPH	benign prostatic hyperplasia
CAH	congenital adrenal hyperplasia
CAH1	*see* 21OHD
cAMP	cyclic adenosine monophosphate
CBG	corticosteroid-binding globulin
CE	collision energy
CRH	corticotropin-releasing hormone
CRPC	castration-resistant prostate cancer
CV	cone voltage
CYP	cytochrome P450
DMEM	Dulbecco's Modified Eagle's Medium
ER	estrogen receptor
ESI+	positive electrospray ionisation
FBS	fetal bovine serum
FSH	follicle-stimulating hormone
GC-MS	gas chromatography-mass spectrometry
GnRH	gonadotropin-releasing hormone

GR	glucocorticoid receptor
hCG	human chorionic gonadotropin
Heat shock protein	hsp
HEK-293	human embryonic kidney cells
HPA	hypothalamus-pituitary-adrenal
LB	Luria-Bertani
LC-MS	liquid chromatography-mass spectrometry
LH	luteinizing hormone
LOD	limit of detection
LOQ	limit of quantification
Mr	molecular weight
MR	mineralocorticoid receptor
MRM	multiple reaction monitoring
MS	mass spectrometry
MTBE	methyl tert-butyl ether
N_2	nitrogen gas
NADH or NAD^+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
nSW	non-salt-wasting
PAPS	3-phospo-adenosine-5'-phosphosulfate
PCa	prostate cancer
pCIneo	vector without insert
PCOS	polycystic ovary syndrome
Pen-Strep	penicillin-streptomycin
PKA	protein kinase A
PR	progesterone receptor
PR-A	progesterone receptor isoform A
PR-B	progesterone receptor isoform B
RAAS	renin-angiotensin-aldosterone system
RPMI-1640	Roswell Park Memorial Institute 1640 medium
RT	retention time

SFC	supercritical fluid chromatography
SHBG	sex hormone-binding globulin
SHBP	steroid hormone-binding protein
T_0	timepoint zero
TART	testicular adrenal rest tumour
UHPLC-MS/MS	ultra-high-performance liquid chromatography tandem mass spectrometry
UPC^2-MS/MS	ultra-performance convergence chromatography tandem mass spectrometry
ZF	zona fasciculata
ZG	zona glomerulosa
ZR	zona reticularis

Enzymes

11βHSD	11β-hydroxysteroid dehydrogenase
11βHSD1	11β-hydroxysteroid dehydrogenase type 1
11βHSD2	11β-hydroxysteroid dehydrogenase type 2
17βHSD	17β-hydroxysteroid dehydrogenase
17βHSD1	17β-hydroxysteroid dehydrogenase type 1
17βHSD2	17β-hydroxysteroid dehydrogenase type 2
17βHSD3	17β-hydroxysteroid dehydrogenase type 3
17βHSD5	17β-hydroxysteroid dehydrogenase type 5
17βHSD6	17β-hydroxysteroid dehydrogenase type 6
3αHSD	3α-hydroxysteroid dehydrogenases
3αHSD1	3α-hydroxysteroid dehydrogenase type 1
3αHSD2	3α-hydroxysteroid dehydrogenase type 2
3αHSD3	3α-hydroxysteroid dehydrogenase type 3
3αHSD4	3α-hydroxysteroid dehydrogenase type 4
3βHSD2	3β-hydroxysteroid dehydrogenase type 2
ADX	adrenodoxin
AKR	aldo-keto reductase

AKR1C2	*see* 3αHSD3
AKR1C3	*see* 17βHSD5
CYP11A1	cytochrome P450 cholesterol side-chain cleavage
CYP11B1	cytochrome P450 11β-hydroxylase
CYP11B2	cytochrome P450 aldosterone synthase
CYP17A1	cytochrome P450 17α-hydroxylase/17,20-lyase
CYP19A1	cytochrome P450 aromatase
CYP21A2	cytochrome P450 21-hydroxylase
cyt b_5	cytochrome b_5
G6PDH	glucose-6-phosphate dehydrogenase
H6PDH	hexose-6-phosphate dehydrogenase
HSD	hydroxysteroid dehydrogenases
P450c17	*see* CYP17A1
PAPSS	3-phospo-adenosine-5'-phosphosulfate synthases
POR	cytochrome P450 oxidoreductase
RL-HSD	retinol-like hydroxysteroid dehydrogenase
RODH	retinol dehydrogenase
SDR	short-chain dehydrogenase/reductase
SRD5A	5α-reductases
SRD5A1	5α-reductase type 1
SRD5A2	5α-reductase type 2
StAR	steroidogenic acute regulatory protein
STS	sulfatases
SULT	sulfotransferases
SULT2A1	sulfotransferase 2A1
UGT	uridine-diphosphate glucuronosyltransferases

Steroid hormones

11-deoxycortisol	4-pregnen-17α,21-diol-3,20-dione
11-DHC	11-dehydrocorticosterone

11K-3αAdiol	11-ketoandrostanediol/5α-androstan-11-one-3α,17β-diol
11K-5αDione	11-ketoandrostanedione/5α-androstan-3,11,17-trione
11KA4	11-ketoandrostenedione/4-androsten-3,11,17-trione
11KAST	11-ketoandrosterone/5α-androstan-3α-ol-11,17-dione
11K-DHP4	11-ketodihydroprogesterone /5α-pregnan-3,11,20-trione
11KDHT	11-ketodihydrotestosterone /5α-androstan-17β-ol-3,11-dione
11KP4	11-ketoprogesterone /4-pregnen-3,11,20-trione
11K-Pdiol	5α-pregnan-3α,17α-diol-11,20-dione
11K-Pdione	5α-pregnan-17α-ol-3,11,20-trione
11KT	11-ketotestosterone /4-androsten-17β-ol-3,11-dione
11OH-3αAdiol	11β-hydroxyandrostanediol/5α-androstan-3α,11β,17β-triol
11OH-5αDione	11β hydroxyandrostanedione/5α-androstan-11β-ol-3,17-dione
11OHA4	11β-hydroxyandrostenedione /4-androstene-11β-ol-3,17-dione
11OHAST	11β-hydroxyandrosterone/5α-androstan-3α,11β-diol-17-one
11OH-DHP4	5α-pregnan-11β-ol-3,20-dione/11β-hydroxy-dihydroprogesterone
11OH-DHP4	5α-pregnan-11β-ol,3,20-dione
11OHDHT	11β-hydroxydihydrotestosterone/5α-androstan-11β,17β-diol-3-one
11OH-etio	11β-hydroxyetiocholanolone /11OH-etiocholanolone
11OHP4	11β-hydroxyprogesterone /4-pregnen-11β-ol-3,20-dione
11OH-Pdiol	5α-pregnan-3α,11β,17α-triol-20-one
11OH-Pdione	5α-pregnan-11β,17α-diol-3,20-dione
11OHT	11β-hydroxytestosterone /4-androsten-11β,17β-diol-3-one
11αOH-DHP4	5α-pregnan-11α-ol-3,20-dione
11αOHP4	11α-hydroxyprogesterone/4-pregnen-11α-ol-3,20-dione
11βOHANDRO	*see* 11OHAST
11βOH-DHP4	*see* 11OH-DHP4
11βOHETIO	*see* 11OH-etio
11βOHP4	*see* 11OHP4
11βOH-Pdione	5α-pregnan-11β,17α-diol-3,20-dione
11βOH-Pdione	*see* 11OH-Pdione
16OHP4	16α-hydroxyprogesterone/4-pregnen-16α-ol-3,20-dione

17OHP4	17α-hydroxyprogesterone /4-pregnen-17α-ol-3,20-dione
17OHP5	17α-hydroxypregnenolone/5-pregnen-3β,17α-diol-20-one
18OH-CORT	18-hydroxycorticosterone/4-pregnen-11β,18,21-triol-3,20-dione
18OH-DOC	18-hydroxy-DOC
20αOHP4	20α-hydroxyprogesterone
21dE	21-deoxycortisone/4-pregnen-17α-ol-3,11,20-trione
21dF	21-deoxycortisol4-pregnen-11β,17α-diol-3,20-dione
3,11diOH-DHP4	5α-pregnan-3α,11β-diol-20-one
3αAdiol	androstanediol/5α-androstane-3α,17β-diol
5αDione	5α-androstanedione/5α-androstan-3,17-dione
5α-THF	*see* THF
5β-THF	5β-tetrahydrocortisol
A4	androstenedione /4-androsten-3,17-dione
A5	androstenediol
ALDO	aldosterone/4-pregnen-11β,21-diol-3,18,20-trione
Alfaxalone	5α-pregnan-3α-ol-11,20-dione
Allo	5α-pregnan-3α-ol-20-one
AST	androsterone/5α-androstan-3α-ol-17-one
CORT	corticosterone/4-pregnen-11β,21-diol-3,20-dione
cortisol	4-pregnene-11β, 17α,21-triol-3,20-dione
cortisone	4-pregnen-17,21-diol-3,11,20-trione
D2-T	deuterated testosterone 1, 2-D2
D3-11KDHT	deuterated 11-ketodihydrotestosterone 16,16,17A
D3-11KT	deuterated 11-ketotestosterone 16,16,17A-D3
D4-cortisol	9, 11, 12,12-D4-cortisol
D5-11K-Etiocholanolone	deuterated 11-keto-etiocholanolone 9A,12,12,16,16-D5
D7-11OHA4	deuterated 11β-hydroxyandrostenedione 2,2,4,6,6,16,16-D7
D7-A4	4-androstene-3,17-dione 2,2,4,6,6,16,16-D7
D8-17OHP4	deuterated 17α-hydroxyprogesterone 2,2,4,6,6,21,21,21-D8
D8-21dF	deuterated 21-deoxycortisol 2,2,4,6,6,21,21,21
D9-P4	deuterated progesterone 2,2,4,6,6,17A,21,21,21-D9

Dex	dexamethasone
DHEA	dehydroepiandrosterone/5-androsten-3β-ol-17-one
DHEAS	DHEA-sulfate
DHP4	dihydroprogesterone/5α-pregnan-3,20-dione
DHT	dihydrotestosterone/5α-androstan-17β-ol-3-one
DHT-G	DHT-glucuronide
DOC	deoxycorticosterone/4-pregnen-21-ol,3,20-dione
Mib	Mibolerone
P4	progesterone /4-pregnen-3,20-dione
P5	pregnenolone /5-pregnen-3β-ol-20-one
Pdiol	5α-pregnan-3α,17α-diol-20-one
Pdione	5α-pregnan-17α-ol-3,20-dione
Prednisone	1,4-pregnadien-17,21-diol-3,11,20-trione
R5020	promegastone
T	testosterone /4-androsten-17β-ol-3-one
THF	tetrahydrocortisol /3α,11β,17α,21-tertahydroxy-5β-pregnan-20-one

Chapter 1

General introduction

In the last decade the C11-oxy C_{19} and C11-oxy C_{21} steroids –steroids containing a keto or hydroxyl moiety at carbon 11, have been reported more frequently *in vivo* underscoring C11-oxy androgens to be as potent as the classical androgens such as testosterone (T) and dihydrotestosterone (DHT). Furthermore, reported levels of adrenal 11β-hydroxyandrostenedione (11OHA4) exceed that of androstenedione (A4) and T (Rege *et al.* 2013) and the C11-oxy C_{19} steroids have since shown as major androgens in both healthy and pathophysiological study subjects (Du Toit and Swart 2018, 2020, Du Toit *et al.* 2018, Rege *et al.* 2018, Nanba *et al.* 2019) with 11-ketodihydrotestosterone (11KDHT), at 1 nM, able to activate the androgen receptor (AR) to the same extent as DHT. An increasing number of research groups are focusing on or including these C11-oxy C_{19} steroids in their current studies aiming to elucidate their biological significance. 11β-hydroxyandrosterone (11OHAST) is, for example, the main urinary metabolite detected in 21-hydroxylase deficiency (21OHD) patients with elevated backdoor pathway activity. However, 11OHA4 was not identified as the precursor metabolite (Kamrath *et al.* 2012). 11OHAST and 11-ketoandrosterone (11KAST) have also been detected in benign prostatic hyperplasia (BPH) patients, both in circulation and in prostate tissue. In addition, C11-oxy C_{21} steroid levels were detected to be higher in these patients than the classical C_{21} steroids including progesterone (P4), 17α-hydroxyprogesterone (17OHP4) and even cortisol and cortisone —except for dihydroprogesterone (DHP4) levels which were detected as the major C_{21} steroid followed by 11-ketodihydroprogesterone (11K-DHP4) (Du Toit and Swart 2020). Increased 5α-reductase activity together with elevated C11-oxy C_{21} steroids suggest that other reduced C11-oxy C_{21} metabolites may be present which are not currently analysed in pathophysiological conditions of endocrinologic disorders. In contrast, with the exception of 11OHA4, higher levels of C_{19} and C_{21} steroids were detected in circulation compared to tissue samples which show that circulation would not necessarily provide a true reflection of steroid biosynthesis in tissue where the steroids would exert a localised effect (Bélanger *et al.* 1990, Hanukoglu *et al.* 1990). 11OHA4, a steroid initially reported to be of little importance due to the lack of androgenicity, is now one of the major C_{19} steroids detected in steroid analysis. Interestingly, elevated levels of C11-oxy C_{21} steroids including 11β-hydroxyprogesterone (11OHP4) and 21-deoxycortisol (21dF), albeit in the low

nanomolar range, have been reported in the same studies in which elevated C11-oxy C_{19} steroids were detected (Fiet *et al.* 2017). These C11-oxy C_{21} steroids have, however, not yet been afforded the same consideration as precursors to elevated 11OHAST levels.

The adrenal-specific cytochrome P450 11β-hydroxylase (CYP11B1) and cytochrome P450 aldosterone synthase (CYP11B2) catalyse the biosynthesis of 11OHA4 from A4 (Swart *et al.* 2013) and that of 21dF from 17OHP4 (Barnard *et al.* 2017). Earlier studies investigating the C11-oxy C_{21} steroids in congenital adrenal hyperplasia (CAH) identified both 21dF and 11OHP4 (Gueux *et al.* 1987) with *in vitro* investigations reporting that CYP11B1 and CYP11B2 catalyse the 11β-hydroxylation of P4 to 11OHP4 (Van Rooyen *et al.* 2018). While the production of 11OHA4 was assumed to be a mechanism inactivating A4 thus regulating active androgen production, the steroid is metabolised in the 11OHA4 pathway to potent C11-oxy C_{19} steroids which include 11β-hydroxytestosterone (11OHT), 11-ketotestosterone (11KT) and 11KDHT. 11OHA4 pathway activity has been reported to contribute to the pool of active androgens in prostate cancer (PCa) as well as polycystic ovary syndrome (PCOS) (Du Toit *et al.* 2017, O'Reilly *et al.* 2017). Apart from the conventional and alternative pathways yielding androgens, the backdoor pathway also yields potent androgens. In this pathway the A-ring of 17OHP4, a C_{21} steroids, is reduced and the metabolite subsequently cleaved by cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) to yield androsterone (AST), an inactive C_{19} steroid which can be re-activated to yield DHT. Elevated backdoor pathway activity has been reported in castration-resistant prostate cancer (CRPC), PCOS and 21OHD. Moreover, our research group previously reported the production of 21dF from 17OHP4 and the subsequent reduction of 21dF and its keto-derivative, 21-deoxycortisone (21dE), by the 5α-reductase isozymes (SRD5A1 and SRD5A2) and subsequently by 3α-reductase (3αHSD3). C11-oxy C_{19} steroids were detected as end-products in the metabolism of 21dF in LNCaP cells, a CRPC cell model. Increased backdoor pathway activity in clinical conditions associated with increased C11-oxy C_{19} and C11-oxy C_{21} steroid levels together with the conversion of 21dF and 21dE by steroidogenic enzymes in the backdoor pathway has renew interest in C11-oxy C_{21} steroids.

The C11-oxy C_{21} steroids were therefore investigated, endeavouring to elucidate the metabolism and biological significance by means of *in vitro* enzymatic assays and analysis of steroid levels. It

is hypothesised that the C11-oxy C_{21} steroids are more prevalent than currently reported and are metabolised in a novel C11-oxy backdoor pathway, potentially contributing to the androgen pool.

Technical advances in analytical tools and mass spectrometric analysis, liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS), has led to numerous research groups developing and publishing a broad selection of MS methods for the analysis of small molecules such as steroids. Each method was developed for the analysis of a selected group of steroids which rarely include more than 20 steroids (Dhillon *et al.* 2011, Ke *et al.* 2014, Caron *et al.* 2015, Quanson *et al.* 2016). Due to the novelty of the research presented, development of new MS methods for the identification and quantification of C11-oxy C_{19} steroids as well as C11-oxy C_{21} steroids were required for this study.

The PhD study undertaken will be presented as follow:

Chapter 2 presents a general overview of the steroidogenic pathways and highlights the backdoor pathway leading to the production of androgen, focusing on the enzymes which catalyse the biosynthesis and metabolism of C11-oxy C_{21} steroids. In addition, this chapter briefly reviews the factors which influence the bioavailability of steroids which include the enzymes that catalyse the inactivation and conjugation of steroid hormones and steroid hormone binding proteins as well as providing an overview of the relevant nuclear receptors, the AR and the progesterone receptor (PR) isoforms. Lastly, it addresses the current status of MS analysis, briefly discussing LC-MS, highlighting the advantages of supercritical fluid chromatography as well as the limitations of current published methods.

The aims of the dissertation which are addressed in the chapters to follow are:

- the investigation into the metabolism of C11-oxy C_{21} steroids by steroidogenic enzymes of the backdoor pathways to elucidate the novel C11-oxy backdoor pathway yielding C11-oxy C_{19} steroids;
- the evaluation of the activity of the 11β-hydroxysteroid dehydrogenase (11βHSD) isoforms in the novel C11-oxy backdoor pathway catalysing the interconversion of C11-oxy C_{21} steroid metabolites;

- to determine whether the C11-oxy C_{21} steroids are agonists of human AR and PR isoforms and whether the C11-oxy C_{19} steroids agonist of the PR;
- the development and validation of UPC^2-MS/MS methods used to analyse (*a*) the cohort of steroids of the adrenal pathways, backdoor pathway as well as the C11-oxy C_{21} steroid metabolites; (*b*) the C11-oxy C_{19} and C11-oxy C_{21} steroids; (*c*) the epi-steroid derivatives;
- the quantitation of free C11-oxy steroids in serum and saliva samples of male athletes and construction of comprehensive steroid profiles to elucidate potential biomarkers of doping and/or diseases and disorders.

Chapter 3 unravels the C11-oxy backdoor pathway in a published manuscript showing the *in vitro* biosynthesis of 11OHP4 by CYP11B1 and CYP11B2, and the subsequent oxidation by 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) to yield 11-ketoprogesterone (11KP4) and the 11-keto reduction yielding 11OHP4 by 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1). Both steroids were rapidly converted by SRD5A to 11β-hydroxy-dihydroprogesterone (11OH-DHP4) and 11K-DHP4 and subsequently by 3αHSD yielding 5α-pregnan-3α,11β-diol-20-one (3,11diOH-DHP4) and 5α-pregnan-3α-ol-11,20-dione (alfaxalone). The latter was converted to 11KAST by CYP17A1 showing the final conversion reaction converting C11-oxy C_{21} steroids to C11-oxy C_{19} steroids. The metabolism of 11OHP4 and 11KP4 was subsequently investigated in LNCaP cells in which the detection of 11KDHT highlights a novel pathway yielding potent androgens.

Chapter 4 described the substrate specificity and conversion of 11OHP4, 11KP4, 21dF and 21dE, as well as the 5α- and 5α,3α-reduced metabolites catalysed by CYP17A1 and explores homology modelling of the enzyme with the docking of 11OHP4, 11KP4 and their reduced metabolites in the active pocket of CYP17A1. In addition, an UPC^2-MS/MS method, which was used for the analysis and quantification of the C11-oxy C_{19} and C11-oxy C_{21} steroids, was developed and validated.

Chapter 5 presents data showing the oxidation of the C11-hydroxy C_{21} steroids catalysed by 11βHSD2 as well as the reverse reaction in which 11βHSD1, when co-expressed with hexose-6-phosphate dehydrogenase (H6PDH), catalyses the reduction of the C11-keto C_{21} steroids. In addition, the interconversion of 11KAST and 11OHAST, and 11-ketoandrostanediol

(11K-3αAdiol) and 11β-hydroxyandrostanediol (11OH-3αAdiol) by the 11βHSD isozymes are also included.

Chapter 6 presents the investigation into the transactivation of the human AR and PR isoforms by 11OHP4, 11KP4, 21dF, 21dE and their 5α-reduced metabolites and briefly addresses the limitations with regards to transiently transfected luciferase reporter promotor assays.

Chapter 7 provides *in vivo* evidence of steroids characteristic of the C11-oxy backdoor in male athletes. In the analysis of epi-steroid derivatives of the C_{19} and C11-oxy C_{19} steroids in serum, a novel UPC^2-MS/MS method was developed and validated and is described in the submitted manuscript. Subsequent analyses were undertaken using a second UPC^2-MS/MS method which was developed and validated to analyse the full spectrum of C_{19}, C11-oxy C_{19}, C_{21} and C11-oxy C_{21} steroids for the analysis of serum and saliva samples.

Chapter 8 presents a conclusion and an overview of the findings presented in the dissertation. It also addresses potential future investigations which would contribute to our understanding of the role of C11-oxy C_{21} steroids.

Chapter 2

Literature Review

2.1 Introduction

C11-oxy steroids, particularly C11-oxy C_{19} steroids, have become the subject of interest in many studies due to their androgenicity and role in the progression of androgen-driven clinical conditions, with numerous reports on C11-oxy C_{19} steroids in health and pathophysiological diseases which include, amongst others, prostate cancer (PCa) and polycystic ovary syndrome (PCOS) as well as 21-hydroxylase deficiency (21OHD). Increased reports on C11-oxy C_{19} steroids and the advances made in chromatography-linked mass spectrometry (MS) have had a knock-on effect resulting in more studies incorporating these hormones in steroid analyses and considering these steroids as potential biomarkers of clinical diseases and disorders. Steroid levels and/or steroid ratios are generally used to elucidate steroidogenic pathways and to identify potential biomarkers. However, the C11-oxy C_{21} steroids, which potentially contribute to C11-oxy C_{19} steroids, are still largely overlooked and, if metabolised via an unidentified pathway, may skew data analyses. In addition, potent C11-oxy androgens have come to the attention of athletes and by the sport communities and used to enhance physical performance and appearance. To date there has been no acknowledgement of these steroids being administered as potential anabolic-androgenic exogenous steroids and as such are not regulated by the doping agencies.

C11-oxy steroids are structurally similar to their C_{19} and C_{21} steroid precursors other than the hydroxyl or keto moiety at C11 situated on the C ring of the steroid structure (Figure 2.1). The two C11-oxy C_{21} steroids, 11β-hydroxyprogesterone (11OHP4) and 21-deoxycortisol (21dF), and the C11-oxy C_{19} steroid, 11β-hydroxyandrostenedione (11OHA4) are 11β-hydroxy metabolites of progesterone (P4), 17α-hydroxyprogesterone (17OHP4) and androstenedione (A4), respectively. These three steroids were first detected in the 1950's (Brownie *et al.* 1954, Touchstone *et al.* 1955, Wieland *et al.* 1965, Gueux *et al.* 1985) but have received little interest until recently.

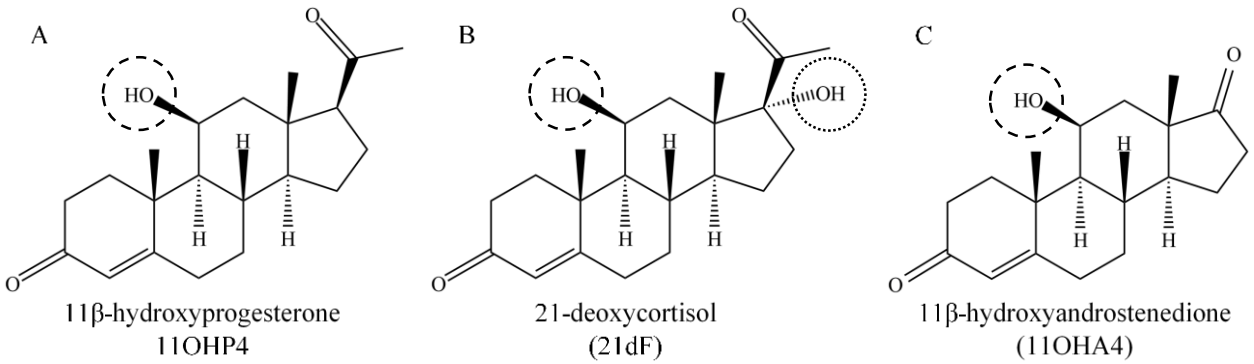

Figure 2. 1 Chemical structure of the adrenal C11-oxy C_{21} steroids and C11-oxy C_{19} steroid. (A) 11β-hydroxyprogesterone (11OHP4), (B) 21-deoxycortisol (21dF) and (C) 11β-hydroxyandrostenedione (11OHA4). The hydroxyl group at C11 on A, B, and C is depicted by the dashed circles and the hydroxyl at C17 of 21dF is shown by dotted circle.

This chapter provides a brief overview of the steroidogenic pathways which include the adrenal steroidogenic pathway together with the conventional-, alternative and backdoor pathways which yield dihydrotestosterone (DHT), the 11OHA4 pathway which yields 11-ketotestosterone (11KT) and 11-ketodihydrotestosterone (11KDHT) as well as the gonadal steroidogenic pathways. The steroidogenic enzymes which catalyse the biosynthesis and metabolism of the C11-oxy C_{19} and C11-oxy C_{21} steroids will be reviewed with specific focus on the backdoor pathway enzymes. Due to the identification of the C11-oxy C_{19} and C11-oxy C_{21} steroids in PCa, PCOS and 21OHD, these will serve as reference clinical conditions and abnormalities with enzyme characteristics pertaining to these diseases and disorders, which may affect the C11-oxy steroid levels.

2.2 Steroidogenic pathways

The following section summarises the biosynthesis of steroids in humans with regards to the adrenal steroidogenic pathway, the various androgen pathways yielding DHT as well as the 11OHA4 pathway in which the C11-oxy C_{19} metabolites are converted to active androgenic compounds. The regulation of the pathways is complex with numerous signalling pathways having been shown to affect the steroid production and/or enzyme expression. This study will address only the most prominent regulatory mechanisms.

Humans have three major endocrine organs which include the adrenal, testis and ovary producing most of the steroids required to maintain steroidogenic homeostasis. Cholesterol, a 27-carbon sterol, is mainly derived in humans from dietary low-density lipoproteins (exogenous) and stored in lipid vesicles as cholesterol esters. Alternatively, cholesterol can be biosynthesised endogenously from acetate in the endoplasmic reticulum, stimulated by adrenocorticotropin-releasing hormone (ACTH). Cholesterol esters are hydrolysed when required for steroidogenesis, and the free form transported from the outer membrane to the inner membrane of the mitochondria by steroidogenic acute regulatory protein (StAR). Cholesterol is metabolised by mitochondrial cytochrome P450 cholesterol side-chain cleavage (CYP11A1) in three reactions (22-hydroxylation, 20-hydroxylation and C20-C22 double bond oxidative scission) leading to pregnenolone (P5) and isocaproaldehyde production (Figure 2.2). High levels of CYP11A1 are expressed in the adrenal cortex, corpus luteum, Leydig cells of the testis and theca cells of the ovary, thus affording these tissues the ability of *de novo* steroidogenesis (Hanukoglu 1992). In addition, CYP11A1 transcripts have been reported in PCa cells lines, with higher levels in the C4-2 cell model relative to LNCaP and VCaP cells, suggesting *de novo* steroid production in diseased tissue (Cai *et al.* 2011).

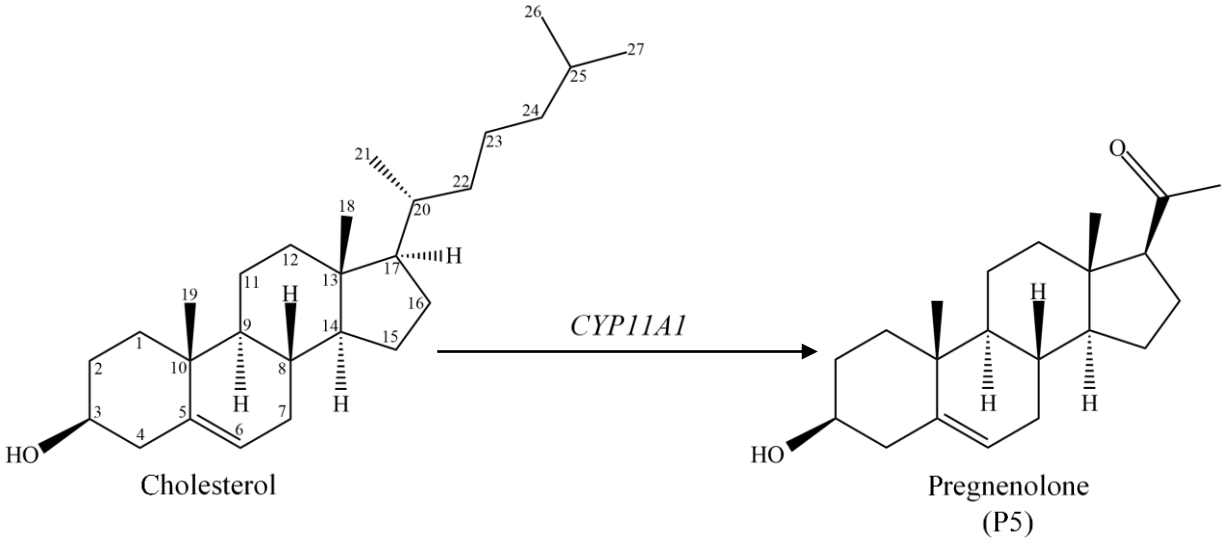

Figure 2. 2 Chemical structures depicting the conversion of cholesterol to pregnenolone catalysed by CYP11A1. Cholesterol is hydroxylated at C22 and 20 followed by the 20,22-lyase reaction ultimately yielding pregnenolone and isocaproaldehyde (not shown).

2.2.1 Adrenal steroidogenesis

The adrenal cortex consists of three functionally distinct zones in which the bulk of the steroids required to regulate and maintain the vast array of physiological processes, are produced. All steroids are biosynthesised from P5 to various end-products depending on the zone and enzyme expression. The zona glomerulosa (ZG) is the outer layer in the adrenal cortex and produces the mineralocorticoids which regulate blood pressure and electrolyte balance (Figure 2.3; magenta). 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2) converts P5 to P4 which is subsequently hydroxylated at C21 in a reaction catalysed by cytochrome P450 21-hydroxylase (CYP21A2) yielding deoxycorticosterone (DOC). Cytochrome P450 aldosterone synthase (CYP11B2) catalyses the 11- and 18-hydroxylation and 18-oxidation steps yielding aldosterone (ALDO) with corticosterone (CORT) and 18-hydroxycorticosterone (18OH-CORT) as intermediates. 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) catalyses the oxidative reaction converting CORT to 11-dehydrocorticosterone (11-DHC), however, adrenal 11βHSD2 activity in the adrenal cortex is low (Mazzocchi *et al.* 1998) while elevated in clinical conditions such as in Cushing's disease (Mune *et al.* 2003). However, CORT can be released into circulation and oxidised by peripheral 11βHSD2 under normal circumstances.

In the zona fasciculata (ZF), which lies between the ZG and the zona reticularis (ZR), P5 is converted to P4 by 3βHSD2 which are both substrates for the 17α-hydroxylase activity of cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) yielding 17α-hydroxypregnenolone (17OHP5) and 17OHP4, respectively, (Figure 2.3; cyan). In addition, human CYP17A1 can hydroxylate P4 at C16 yielding 16α-hydroxyprogesterone (16OHP4) resulting in a 16OHP4:17OHP4 of 1:4 (Swart *et al.* 1993). 17OHP5 is also converted to 17OHP4 catalysed by 3βHSD2. CYP21A2 catalyses the conversion of P4 and 17OHP4 yielding DOC and 11-deoxycortisol followed by the cytochrome P450 11β-hydroxylase (CYP11B1) catalysed hydroxylation to CORT and cortisol, respectively.

Adrenal androgen precursors are produced in the inner ZR layer in the Δ^5-androgen pathway (Figure 2.3; black) by 3βHSD2, CYP17A1 and cytochrome b_5 (cyt b_5) —a co-enzyme which enhances the lyase activity of CYP17A1 (Dharia *et al.* 2004, Nakamura *et al.* 2011). Due to the lack of CYP21A2 expression, P5 and P4 are hydroxylated by CYP17A1 and 17OHP5 is subsequently converted to dehydroepiandrosterone (DHEA) by the 17,20-lyase activity of

CYP17A1. DHEA, a major androgen precursor, is further metabolised by 3βHSD2 to yield A4 or reduced at C17 by 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5 a.k.a AKR1C3) to androstenediol (A5). However, adrenal expression of 17βHSD5 is low (Hofland *et al.* 2013) and thus not a dominant reaction in the adrenal producing only low levels of A5 and testosterone (T) (Nakamura *et al.* 2009). It should also be noted that, in the adrenal, most of the DHEA biosynthesised is sulfated by sulfotransferase 2A1 **(**SULT2A1) (Auchus and Rainey 2004). In humans, 17OHP4 is a poor substrate for the lyase activity of CYP17A1 resulting in negligible A4 biosynthesis via the Δ^4-pathway. In addition, the adrenal produces high levels of 11OHA4 as well as low levels of 11KT, 11-ketoandrostenedione (11KA4), and 11β-hydroxytestosterone (11OHT) (Rege *et al.* 2013, Swart *et al.* 2013). 11OHA4 is biosynthesised from the 11β-hydroxylation of A4 catalysed by CYP11B1 and while CYP11B2 has been shown to catalyse the same reaction *in vitro* (Swart *et al.* 2013) the latter is perhaps not physiologically relevant due to CYP11B2's expression being restricted to the ZG.

The biosynthesis of adrenal steroids is regulated by extracellular hormones which include the hypothalamic corticotropin-releasing hormone (CRH) which signals the pituitary to release ACTH which, in turn, stimulates the adrenal via the hypothalamus-pituitary-adrenal (HPA) axis. ACTH binds ACTH receptors in the ZF and ZR (Reincke *et al.* 1998), leading to the production of cyclic adenosine monophosphate (cAMP) which in turn regulates cell growth, stimulates the release of cholesterol and modulates enzyme expression (Miller and Auchus 2011). The expression of CYP11A1, CYP17A1, CYP21A2, CYP11B1 and, to a lesser extent, CYP11B2 is regulated by ACTH (Clark and Cammas 1996). In contrast, enzyme expression in the ZG is regulated by the renin-angiotensin-aldosterone system (RAAS) with angiotensin II (AngII) stimulating *de novo* mineralocorticoid steroidogenesis either by increasing cellular cholesterol and cofactor levels in response to acute stimuli, or by upregulating enzyme expression in response to chronic stimuli.

It is important to note that the adrenal's hormonal contribution to the endocrine system is primarily mineralocorticoid and glucocorticoid hormones with sex steroid biosynthesis more prominent in steroidogenic tissue. Limited sex steroid production in the adrenal is partly due to low expression levels of the 17βHSD isozymes with 5α-reductases (SRD5A) and cytochrome P450 aromatase (CYP19A1) expression in the gonads catalysing the production of estrogens and active androgens. However, as mentioned previously most of the DHEA produced in the adrenal is sulfated by

SULT2A1, expressed in the ZR, and secreted as DHEA-sulfate (DHEAS) into circulation. Rege *et al*. (2013) analysed adrenal vein steroid levels in seven women with primary aldosteronism and detected DHEAS as the most abundant adrenal C_{19}, pre- and post-ACTH stimulation, 30-fold and 6-fold higher than DHEA, respectively (Rege *et al.* 2013). DHEAS is subsequently released into circulation, with levels reported to range between 2-10 µmol/L in males and 1-8 µmol/L in females (Mueller *et al.* 2015), and transported to target tissue where sulfatases remove the sulfate group rendering DHEA free too be converted to A4 and subsequently potent sex steroids. Both DHEAS and DHEA therefore present as precursor steroids in androgen biosynthesis pathways leading to the production of active androgen. These pathways, which include both the conventional and alternative pathway, therefore utilize precursors of adrenal origin leading to the production of DHT. DHT is, however, also biosynthesised in the backdoor pathway in which 17OHP4 is the precursor steroid. While the adrenal does produce very low levels of T, 11OHA4 is one of the major C_{19} steroids produced in the adrenal. 11OHA4 contributes to the pool of active androgens as it is metabolised in the periphery to active androgens 11KT and 11KDHT in the 11OHA4 pathway.

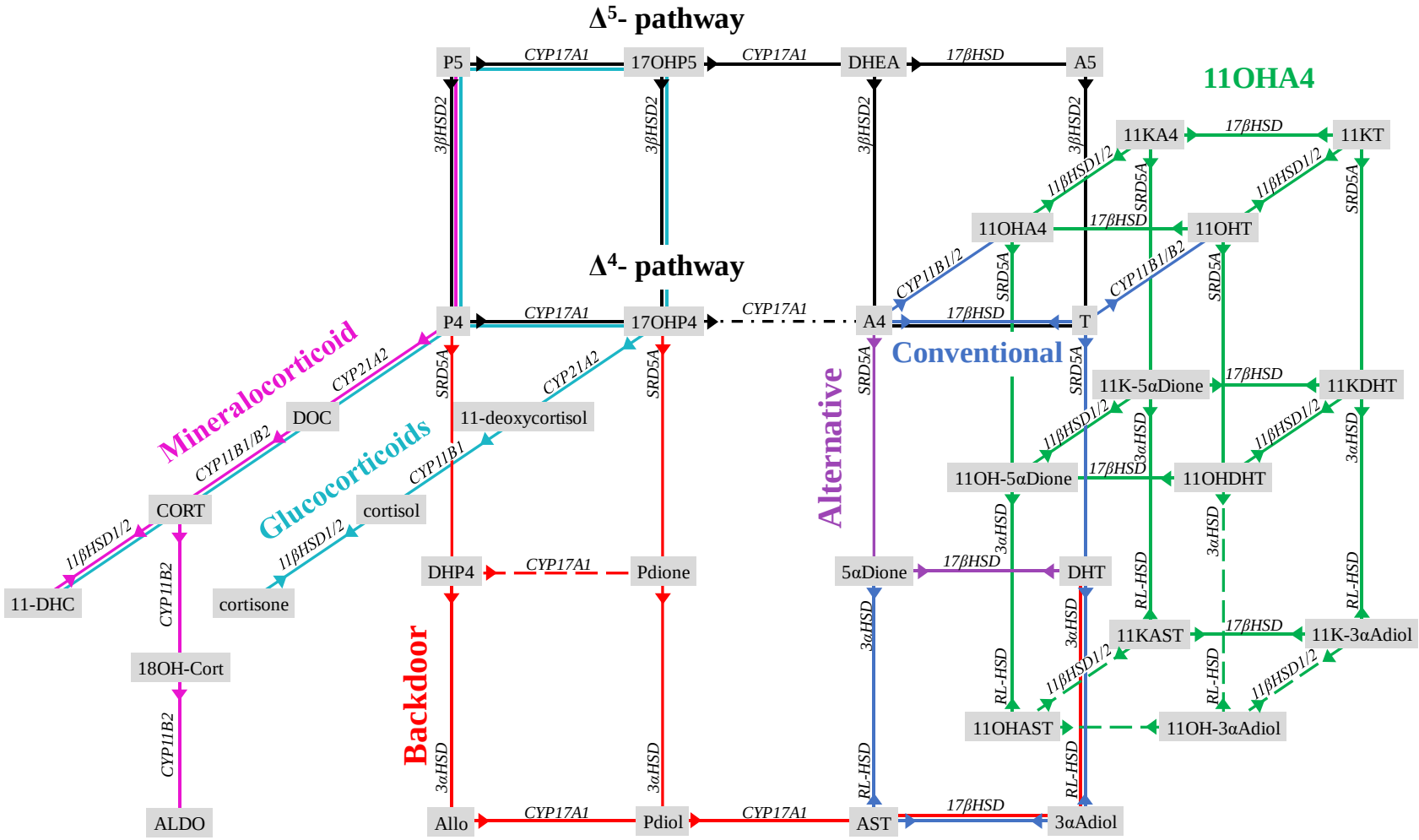

Figure 2. 3 Schematic representation of the major steroidogenic pathways and peripheral metabolic reactions. Adrenal steroidogenesis includes the Δ^5 and Δ^4-pathway (black), mineralocorticoid- (magenta) and glucocorticoid pathway (cyan) and peripheral 11βHSD conversions of cortisol and cortisone, and CORT and 11-DHC is shown. Androgen pathways to DHT include the conventional- (blue), alternative- (purple) and backdoor pathway (red). The 11OHA4 pathway is shown in green. Dashed lines represent potential reaction which have, to date, not been elucidated and dash-dot line shows negligible conversion in humans.

2.2.2 Conventional and alternative androgen pathways

DHT is a potent androgen which acts via the androgen receptor (AR) initiating androgen-responsive mechanisms important in male sexual development and differentiation, puberty as well as in reproduction. Although adrenal steroidogenesis can contribute to the androgen pool with the production of A4, T and DHEA, the levels are too low to sustain masculinization or for estrogen biosynthesis required in females. The testis and ovaries, specifically the Leydig cells of the testis and theca cells of the ovary, biosynthesise the bulk of androgens required in physiological homeostasis. Both these tissues express CYP11A1 and can thus biosynthesise the aforementioned steroids *de novo*. The enzyme expression profile resembles that of the ZR in the expression of 3βHSD2, CYP17A1, cyt b_5 however, with higher levels of 17β-hydroxysteroid dehydrogenases (17βHSD). P5 is thus metabolised by CYP17A1 to DHEA which is converted to A4, catalysed by 3βHSD2. In the conventional pathway (Figure 2.3; blue), the C17-keto moiety of A4 is reduced by 17βHSD5 and 17βHSD type 3 (17βHSD3) in the testis, yielding T which is subsequently reduced by the SRD5A to DHT.

In the alternative pathway (Figure 2.3; purple), DHT is biosynthesised from A4 without requiring T as an intermediate. Instead, A4 is firstly converted to 5α-androstanedione (5αDione) by SRD5A followed by the 17βHSD-catalysed reduction to DHT (Chang *et al.* 2011, Sharifi and Auchus 2014). In addition, the expression of the hydroxysteroid dehydrogenases (HSD) regulate the bioactivity of C_{19} steroids by catalysing the interconversion of DHT and 3αAdiol, and 5αDione and androsterone (AST) —fluctuating between the active and inactive steroid metabolites unless conjugated. In castration-resistant prostate cancer (CRPC) the alternative pathway dominates due to elevated prostatic SRD5A expression and lack of testicular T resulting in the uptake of A4 in the pathway to re-equilibrate DHT levels.

2.2.3 Backdoor pathway

In 2004, Auchus *et al.* proposed a backdoor pathway to DHT production from androstanediol (3αAdiol), circumventing A4 and T production, after observations made in the testis of young tammar wallabies and immature mice (Auchus 2004). The backdoor pathway (Figure 2.3; red), which is of particular interest to this study, has since been implicated in hyperandrogenic disorders which include PCOS (Marti, Galvan, *et al.* 2017) and 21OHD (Kamrath, Hochberg, *et al.* 2012),

and diseases such as PCa. The C4-C5 double bond of the C_{21} steroids, P4 and/or 17OHP4, are reduced by the SRD5A isozymes to yield 5α-pregnan-3,20-dione (DHP4) and 5α-pregnan-17α-ol-3,20-dione (Pdione), and are subsequently reduced to 5α-pregnan-3α-ol-20-one (Allo) and 5α-pregnan-3α,17α-diol-20-one (Pdiol) catalysed by 3α-hydroxysteroid dehydrogenase type 3 (3αHSD3 or AKR1C2), respectively. CYP17A1 catalyses the 17α-hydroxylation of Allo to Pdiol, which is rapidly converted to AST by the 17,20 lyase activity of the enzyme and which in turn, can be converted by HSD to ultimately yield DHT. Although Gupta *et al.* (2003) showed the 17α-hydroxylation of DHP4 to Pdione, the data lacked a positive control for the identification of the product (Gupta *et al.* 2003).

Furthermore, both the backdoor- and the conventional pathway were reported to be a prerequisite in the differentiation of male genitalia (Biason-Lauber *et al.* 2013) with AST detected as the major C_{19} steroid in circulation of male foetuses with elevated backdoor pathway enzymes transcripts detected in non-gonadal tissue (O'Shaughnessy *et al.* 2019). Further evidence of these pathways *in vivo* was reported by Marti *et al.* (2017) who showed the full complement of steroidogenic backdoor pathway enzymes in ovarian theca cells together with elevated expression levels of these enzymes in PCOS (Marti, Galvan, *et al.* 2017).

2.2.4 11OHA4 pathway

11OHA4 is an adrenal steroid biosynthesised from A4 in the conversion of DHEA catalysed by 3βHSD2 and subsequently by CYP11B, which hydroxylates A4 at C11. Since its detection in 1955 (Touchstone *et al.* 1955), and studies showing that 11OHA4 exhibits low androgenic activity (Bélanger *et al.* 1993, Bloem *et al.* 2015), the steroid has become the topic of numerous studies which recently led to the elucidation of the 11OHA4 pathway in which 11OHA4 is the precursor to a number of C11-oxy C_{19} steroids (Figure 2.3; green). Steroidogenic enzymes catalysing the reactions leading to the C11-oxy C_{19} metabolites include CYP11B1, SRD5A, 3α-hydroxysteroid dehydrogenases (3αHSD) isozymes, 17βHSD isozymes, retinol-like hydroxysteroid dehydrogenase (RL-HSD) as well as 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) and 11βHSD2.

11OHA4 is reduced at C11 and C17 by 11βHSD1 and 17βHSD3/5, respectively, to yield 11KA4 and subsequently 11KT in the 11OHA4 pathway. In addition, 11OHT is biosynthesised from the

reduction of 11KT by 11βHSD1 and hexose-6-phosphate dehydrogenase (H6PDH). While 11OHA4 is not a substrate for 17βHSD5 and a poor substrate for 17βHSD3, resulting in negligible 11OHT production (Storbeck *et al.* 2013, Barnard *et al.* 2018), 11OHT is readily converted back to 11OHA4, catalysed by 17βHSD type 2 (17βHSD2) (Swart *et al.* 2013). 11βHSD2 is expressed at low levels in the adrenal, which converts 11OHA4 to 11KA4 and 11OHT to 11KT. However, negligible levels of 11KA4, 11KT, and 11OHT are biosynthesised in the adrenal (Rege *et al.* 2013, Turcu *et al.* 2016). Alternatively, adrenal 11OHA4 is released into circulation and subsequently metabolised via the 11OHA4 pathway in gonadal and peripheral tissue. The 5α-reduction of 11OHA4, 11KA4, 11OHT, and 11KT produces steroid intermediates, 11β-hydroxyandrostanedione (11OH-5αDione), 11-ketoandrostanedione (11K-5αDione), 11β-hydroxydihydrotestosterone (11OHDHT) and 11KDHT, respectively. 11βHSD isozymes catalyse the interconversion of the C11-hydroxyl and C11-keto derivative, while 17βHSD catalyses the oxidation of 11OHDHT and 11KDHT to 11OH-5αDione and 11K-5αDione, respectively and the reduction of 11K-5αDione back to 11KDHT. The four intermediates are converted by 3αHSD, which reduces the keto group at C3 yielding 11β-hydroxyandrosterone (11OHAST), 11-ketoandrosterone (11KAST), 11β-hydroxyandrostanediol (11OH-3αAdiol), and 11-ketoandrostanediol (11K-3αAdiol). These metabolites are interconverted by the 17βHSD isozymes with 3αHSD3 and 17βHSD2 catalysing the interconversion between 11KAST and 11K-3αAdiol. 11OHAST is not converted to 11OH-3αAdiol *in vitro* with the reverse reaction catalysed by 17βHSD2 not having been shown. Barnard *et al.* (2018) has shown that the C11-keto steroids are the preferred substrate for 17βHSD5 which converts 11KA4, 11K-5αDione and 11KAST to 11KT, 11KDHT, and 11K-3αAdiol, respectively (Barnard *et al.* 2018) —and as a consequence directing steroidogenesis towards C11-keto production.

2.2.5 Gonadal steroidogenesis

Steroidogenic enzymes and mechanism of regulation distinguishes the testicular steroidogenesis from the adrenal Δ^5-pathway. In the adrenal the presence of cyt b_5 in the ZR regulates the biosynthesis to androgen production in the presence of CYP17A1 whereas in the ZF, the lack of cyt b_5 directs steroidogenesis towards glucocorticoid production. Leydig cells of the testis express all the necessary steroidogenic enzymes required for *de novo* T biosynthesis, which include CYP11A1, CYP17A1, 3βHSD2, and 17βHSD3 (Geissler *et al.* 1994, Andersson *et al.* 1995, Payne

and Hales 2004) (Figure 2.3; black). In Leydig cells P5 is thus catalysed by CYP17A1, with the conversion to DHEA enhanced by cyt b_5, which in turn, is converted to A5 by 17βHSD3. DHEA and A5 are subsequently converted to A4 and T, respectively, catalysed by 3βHSD2. In addition, P4 catalysed by 3βHSD2 is converted to 17OHP4 to yield low levels of A4.

T production in the testis is regulated by various factors. The most prominent regulatory system includes the negative feedback mechanism in which the hypothalamus, stimulated by low T levels, releases gonadotropin-releasing hormone (GnRH) which in turn signals the anterior pituitary to release luteinizing hormone (LH). The binding of LH to the LH receptors located on the Leydig cell surface activates adenylate cyclase leading to cAMP biosynthesis which subsequently activates various kinases which include protein kinase A (PKA) leading to transcriptional activation (Tremblay 2015). In addition, AR signalling in the adult testis plays an important role in gene-transcriptional regulation resulting in T biosynthesis (Andric and Kostic 2019). Since Leydig cells express low levels of SRD5A, the conversion of T to DHT in men occurs mainly in the prostate which also expresses 17βHSD type 2, type 3 and type 5 (Thigpen *et al.* 1993, Payne and Hales 2004).

Female gonadal steroidogenesis is more complex with the ovaries consisting of two cell types, the theca and granulosa cells —each exhibiting unique enzyme expression profiles. *De novo* steroidogenesis occurs in the theca cells which expresses all the necessary enzymes to metabolise P5 to A4 and T in the Δ^5 pathway (Figure 2.3; black). Androgen precursors diffuse into the granulosa cells where CYP19A1 and 17βHSD1 are expressed catalysing the conversion to yield estrogen (not shown). Gene-transcription in the ovary is regulated by LH and follicle-stimulating hormone (FSH). LH binds to the LH receptor expressed in the theca cells which activate kinases mediated by second messenger systems resulting in C_{19} biosynthesis such as A4 and T, while FSH stimulates CYP19A1 in the granulosa cells (Lew 2019). Although not all are addressed in this review, it is important to note that numerous signalling pathways regulate gonadal steroidogenesis (Wood and Strauss 2002, Andric and Kostic 2019).

Further contributing to the complexities of steroidogenesis is the ever-changing enzyme expression profiles throughout various developmental stages, perfectly highlighted in fetal steroidogenesis — and in this context, the role of the placenta is a major endocrine organ, albeit a temporary one! It is evident that steroidogenic pathways discussed above cannot be considered in isolation, and it

should be noted that complex metabolic routes in which steroids are metabolised are present in tissue other than the adrenal and gonads —in peripheral steroidogenic tissue, endocrine organs such as the placenta and skin, in adipose tissue, in the brain and kidney, to name but a few, which fall beyond the scope of this thesis. Nevertheless, the production of intermediates and specific end-products is governed by tissue-specific enzymes and their expression levels which ultimately contribute to complex steroid profiles unique to the endocrine organ or steroidogenic tissue.

2.3 Steroidogenic enzymes

The abovementioned pathways are the major steroidogenic pathways in various steroidogenic tissue in humans, and steroids, once released into circulation, can be further metabolised upon reuptake. Enzyme expression levels, substrate specificity and competition for substrates influence the steroidogenic outcome. The following section will briefly review the steroidogenic enzymes of interest to this study which catalyse the biosynthesis and metabolism of C11-oxy C_{21} and C11-oxy C_{19} steroids, their substrate specificity, expression and regulation as well as changes observed in clinical conditions such as PCa, PCOS and 21OHD.

2.3.1 Cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1)

Once cholesterol has been converted to P5 by CYP11A1, common to all steroidogenic pathways, P5 is either converted by 3βHSD or CYP17A1. CYP17A1 plays a central critical role in the adrenal where the enzyme is at a branchpoint determining precursor steroids to be channelled into either androgen, mineralocorticoid- or glucocorticoid production while in steroidogenic tissue it catalyses the ultimate production of downstream sex steroid precursors.

CYP17A1 is a microsomal enzyme and catalyses the conversion of P5 and P4 in the Δ^5 and Δ^4 pathways (Figure 2.4). In the adrenal, the enzyme is crucial of the biosynthesis of glucocorticoids and adrenal androgen precursors (Figure 2.3). It is mainly expressed in the ZF and ZR of the adrenal (Giatromanolaki *et al.* 2019) and in steroidogenic tissue, in the testis (Leydig cells) (Chung *et al.* 1987) and the ovary (theca cells) (Sasano *et al.* 1989). CYP17A1 has a dual-enzymatic function catalysing the hydroxylation at C17 and the subsequent 17,20 carbon-carbon cleavage of C_{21} steroids from a single active site (Figure 2.5; navy). However, the hydroxylase and lyase reactions can either occur continuously, in which the lyase reaction follows the hydroxylase

reaction, or discontinuously in which the intermediate is first released from the active site and has been extensively reviewed (Porubek 2013). In adrenal and gonadal steroidogenesis, CYP17A1 catalyses the 17α-hydroxylation and 17,20-lyase of P5 to 17OHP5 and DHEA as well as the hydroxylation of P4 to 17OHP4 and 16OHP4 in a 4:1 ratio —the latter reaction unique to humans and chimpanzee due to the presence of an alanine residue at position 105 instead of leucine as in other species (Swart *et al.* 1993). Although CYP17A1 hydroxylates P5 and P4 with equal efficiency, the lyase reaction favours the Δ^5-steroid with a catalytic efficiency 100-fold higher than the lyase reaction of 17OHP4 with negligible A4 production (Auchus *et al.* 1998).

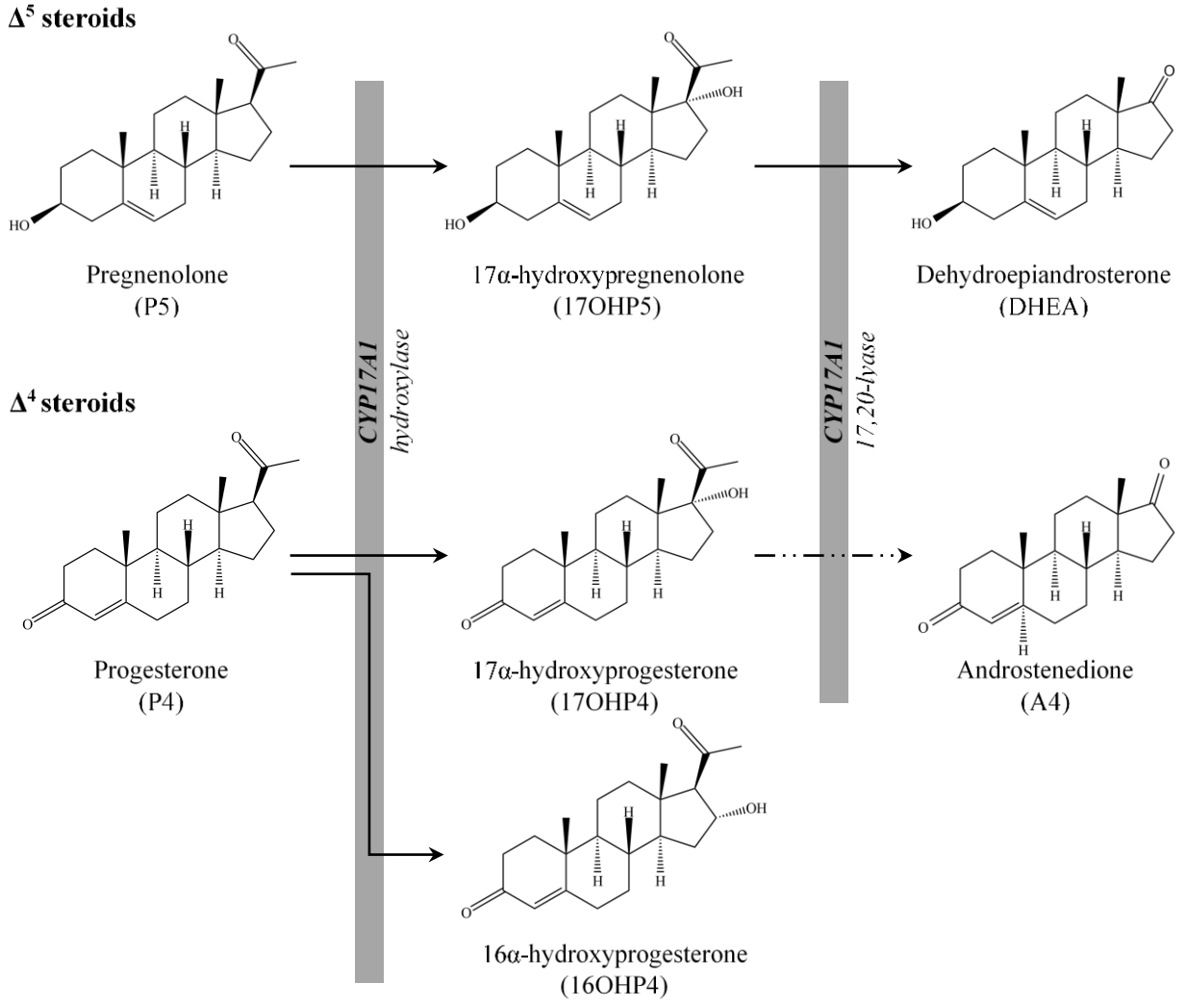

Figure 2. 4 Human CYP17A1 catalyses the conversion of pregnenolone to 17α-hydroxypregnenolone and DHEA and the hydroxylation of progesterone to 17α- and 16α-hydroxyprogesterone. Negligible androstenedione is produced from 17αhydroxyprogesterone in humans.

Although numerous factors can influence the expression of a steroid enzyme, CYP17A1 is mainly regulated by ACTH which bind to ACTH receptors, activating a signalling cascade such as the cAMP-dependent PKA which promotes the transcription of CYP17A1 (Sewer and Jagarlapudi 2009). Interestingly, in adrenal H295R cells, a popular adrenal cell model, CYP17A1 activity was decreased by AngII, which regulates the expression of CYP11B2 (Bird *et al.* 1996).

Furthermore, the regulation of the hydroxylation and lyase activity of CYP17A1 is influenced by protein-protein interactions of cyt b_5 with the membrane-bound, redox partner flavoprotein, cytochrome P450 oxidoreductase (POR) (Auchus *et al.* 1998, Geller *et al.* 1999). While the POR-mediated transfer of electrons, donated from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) to microsomal P450 enzymes is sufficient for the hydroxylase reaction, the lyase reaction requires both cyt b_5 and POR, with an *in vitro* CYP17A1:cyt b_5 ratio of 1:10-30 for optimal function with cyt b_5 acting as a facilitator rather than an electron donor (Auchus *et al.* 1998). Cyt b_5 is a hemeprotein which acts as an allosteric enhancer of CYP17A1-POR activity and increases the conversion of 17OHP4 to A4 and of 17OHP5 to DHEA by 10 and 13-fold, respectively, while the hydroxylation of P5 is only increased by 2-fold (Katagiri *et al.* 1995, Auchus *et al.* 1998). Petrunak *et al.* (2014) reported that even minimal changes in the orientation of 17OHP5 in the active pocket of human CYP17A1, which influenced the distance between asparagine (Asn) at position 202 and the heme group, influenced substrate specificity thus impacting the lyase reaction (Petrunak *et al.* 2014). In addition, anionic glutamic acid residues at positions 48 and 49 of cyt b_5 were shown to interact with the cationic arginine residues at positions 347, 358 and 449 of CYP17A1, located across from the ligand/heme interface, inducing conformational changes in the enzyme's structure and subsequently retaining the ligand and facilitating lyase reaction (Yadav *et al.* 2017).

Cyt b_5 has been reported to be expressed in the ZR of the adrenal cortex (Yanase *et al.* 1998), in the testis (Katagiri *et al.* 1995) and in the ovary (Dharia *et al.* 2004). The regulatory mechanism pertaining to the bifunctional CYP17A1 activity in the presence of POR and cyt b_5 is a complex system and numerous models have been proposed (Raj Bhatt *et al.* 2017). The dual activities of CYP17A1 were also shown to be regulated by post-translational modifications in which serine phosphorylation of CYP17A1 increased only the lyase activity (Pandey *et al.* 2002, Tee *et al.* 2008). Various regulatory mechanisms regarding the CYP17A1-catalysed reactions have been reviewed (Porubek 2013, Fernández-Cancio *et al.* 2018). However, an in-depth discussion is

beyond the scope of the project. In addition, CYP17A1 plays a pivotal role in the backdoor pathway, catalysing the biosynthesis of 17OHP4, the precursor substrate, as well as catalysing the final conversion of Pdiol, a C_{21} steroid, to AST, a C_{19} steroid. Pdiol was reported to be a superior substrate for CYP17A1, which is readily produced by the lyase activity of CYP17A1, regardless of the presence of cyt b_5 which only led to a 3-fold increase when cyt b_5 was present in the assay (Gupta *et al.* 2003). This finding underlies the role of CYP17A1 in androgen-dependent diseases associated with elevated backdoor pathway activity.

Studies have reported the expression of CYP17A1 in PCa cell models such as 22Rv1 and in PCa tissue (Giatromanolaki *et al.* 2019) which normally do not express any CYP17A1. Increased ovarian CYP17A1 expression and activity as well as cyt b_5 mRNA levels detected in theca cells have also been detected in PCOS patients (Qin and Rosenfield 1998, Wood *et al.* 2003, Comim *et al.* 2013). Inhibition of CYP17A1 is thus often a target in the treatment of androgen-dependent diseases using compounds such as ketoconazole, abiraterone acetate and flutamide (Dreicer *et al.* 2014, Harrison *et al.* 2015).

While the biosynthesis of P4 and 17OHP4 in steroidogenic tissue leads to the production of low levels of A4 in humans, with 17OHP4 potentially serving as a substrate for backdoor pathway enzymes once reduced by SRD5A, in the adrenal the Δ^4-pathway steroids are committed to the production of ALDO and cortisol. P4 and 17OHP4 in the ZG and ZF, are substrates for CYP21A2 which catalyse the first reactions in the pathways leading to mineralocorticoid and glucocorticoid pathways.

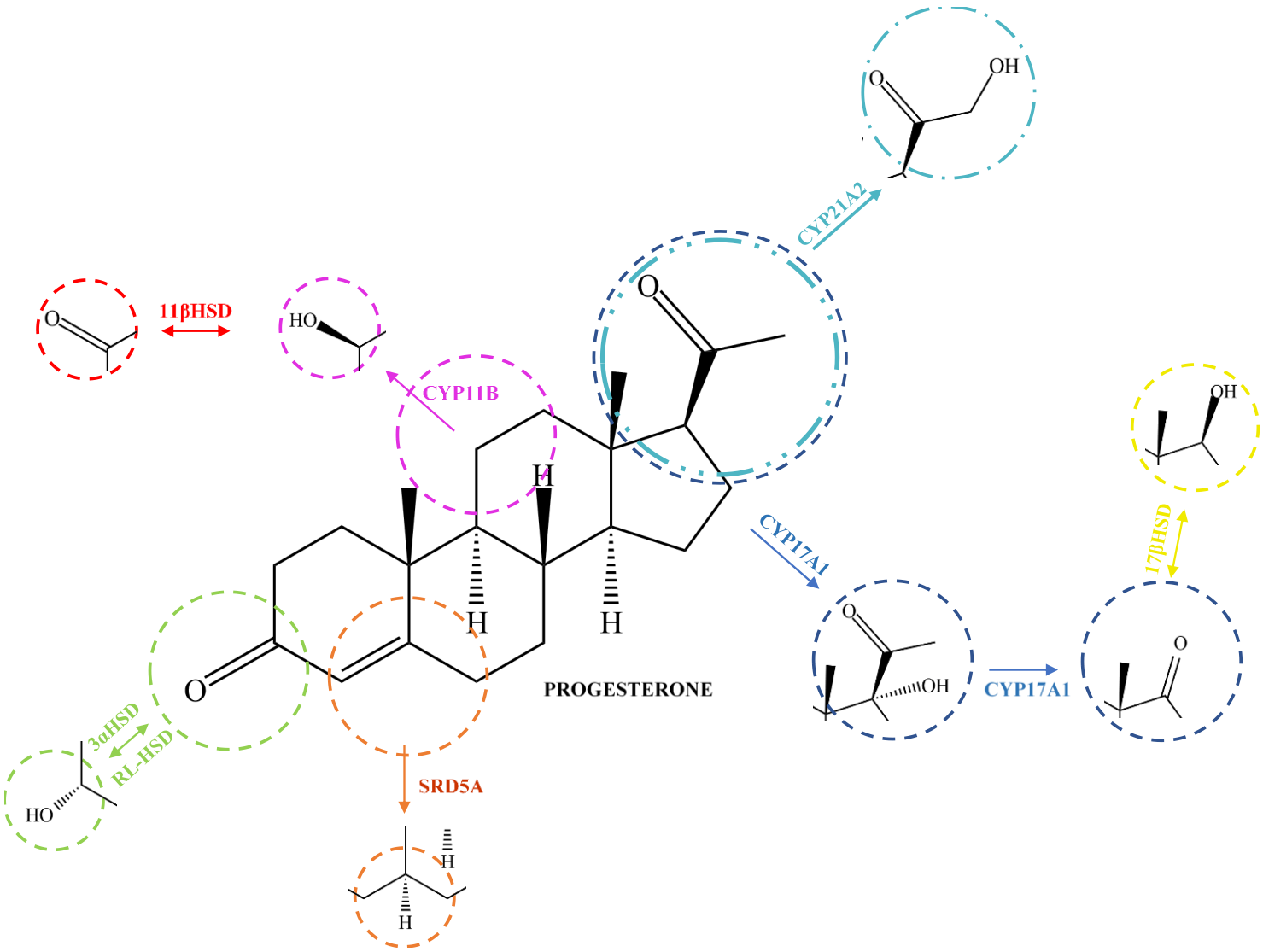

Figure 2. 5 Steroid conversion catalysed by CYP21A2 (blue), CYP17A1 (navy), CYP11B (pink), 11βHSD (red), 17βHSD (yellow), 5α- (orange) and, 3α-reductases and RL-HSD (green). Progesterone is used as an example

2.3.2 Cytochrome P450 21-hydroxylase (CYP21A2)

CYP21A2 is an adrenal-specific microsomal P450 enzyme expressed in the ZG and ZF and catalyses the 21-hydroxylation of Δ^4-steroids (Figure 2.5; blue) while also able to convert certain steroidal xenobiotics (Stoll *et al.* 2019). P4 is converted to DOC in the mineralocorticoid and glucocorticoid pathways and 17OHP4 to 11-deoxycortisol in the glucocorticoid pathway (Figure 2.3; magenta and cyan) with the latter reaction occurring at a higher catalytic efficiency compared to P4 (Tusie-Luna *et al.* 1990). Similar to CYP17A1, the oxidative reaction utilises two electrons from NADPH transported by POR which is widely expressed in various tissue (Figure 2.6) (Miller and Auchus 2011). The adrenal expression of CYP21A2 is regulated by cAMP-activated signalling in response to ACTH (Watanabe *et al.* 1993).

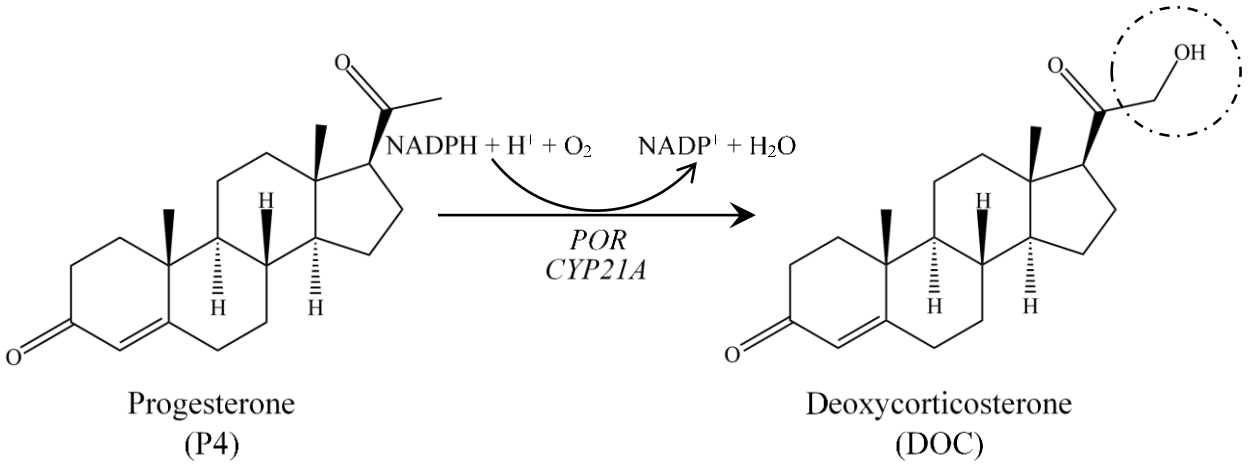

Figure 2. 6 CYP21A2 utilises two electrons donated by NADPH and transported by POR to hydroxylate progesterone (P4) at C21 to yield deoxycorticosterone (DOC).

Deficiency of CYP21A2 is the most common form of congenital adrenal hyperplasia (CAH) which results in a deficiency of mineralocorticoids and glucocorticoids and increased adrenal androgen production which leads to clinical symptoms which include hypotension, virilization of female patients and in serious cases, death (Auchus 2004, Turcu and Auchus 2016, Pignatelli *et al.* 2019). 21OHD can be classified as salt-wasting/severe (>98 % loss of activity), simple virilising (accumulation of 17OHP4 but sufficient ALDO biosynthesis) or nonclassical CAH (mildly dysregulated steroid production). In response to low ALDO and cortisol levels, the HPA axis is stimulated to release ACTH which in turn stimulates adrenal steroidogenesis. However, the lack

of 21-hydroxylase activity results in the accumulation of steroid precursors which are metabolised in the Δ^5-pathway and, together with P4 and 17OHP4 metabolised by the CYP11B enzymes, yield 11OHP4, 21dF and adrenal C_{19} and C11-oxy C_{19} steroids (Turcu *et al.* 2015, 2016). Thus, elevated 17OHP4, 16OHP4, 11OHP4, 21dF, P5-sulfate, A4 and, more recently, C11-oxy C_{19} steroids have been identified as biomarkers in the diagnosis of CAH (New *et al.* 1983, Rege, Turcu, *et al.* 2019).

There are two *CYP21* genes in humans of which the transcription of *CYP21A2*, and not *CYP21A1P* (pseudogene), yields a functional protein. These genes are located in close proximity to each other and recombination of the functional and pseudo *CYP21* genes result in the inactivation or impairment of CYP21A2 activity leading to various 21OHD phenotypes (White and Speiser 2000). Some CYP21A2 mutations result in only partial loss of activity, thus, resulting in decreased mineralocorticoid and glucocorticoid production (Tusie-Luna *et al.* 1990, 1991, Helmberg *et al.* 1992). In addition, it is suggested that low levels of mineralocorticoids detected in 21OHD patients, in which CYP21A1 activity is absent, are biosynthesised by liver 21-hydroxylases such as CYP3A4 and CYP2C19 and subsequently influence the 21OHD phenotype (Miller and Auchus 2011).

Interestingly, it has been shown that high levels of P4 (0.5 -2.0 µM) inhibit the wild-type and non-classical CYP21A2-catalysed 21-hydroxylation of 17OHP4 *in vitro* (Tusie-Luna *et al.* 1990) which can potentially contribute to the accumulation of 17OHP4 even in the presence of low CYP21A1 activity —as in the case of classic simple virilising CAH phenotype in which ALDO levels are normal or elevated while cortisol production is impaired (White and Speiser 2000). The use of abiraterone acetate, a treatment compound used by CRPC patients, decreases androgen biosynthesis by inhibiting CYP17A1. However, CYP21A2 is also inhibited by abiraterone acetate and the 3-oxometabolite of the compound, as have been shown in spectral assays as well as in adrenal H295R cells resulting in impaired glucocorticoid biosynthesis (Malikova *et al.* 2017, Masamrekh *et al.* 2020).

CYP21A1 therefore plays a crucial role in maintaining mineralocorticoid and glucocorticoid homeostasis by biosynthesising DOC and 11-deoxycortisol, the natural substrates for CYP11B2 and CYP11B1. The zonal-specific expression of the CYP11B isozymes and substrate availability are determining factors which mediate the production of mineralocorticoids and glucocorticoid in the adrenal cortex.

2.3.3 Cytochrome P450 11β-hydroxylase (CYP11B1) and cytochrome P450 aldosterone synthase (CYP11B2)

Cytochrome CYP11B1 and CYP11B2 are adrenal-specific enzymes situated on the inner mitochondrial membrane which catalyse reactions in the glucocorticoid and mineralocorticoid pathways yielding cortisol and ALDO, respectively (Miller and Auchus 2011). Unlike the microsomal P450, the electron transfer from NADPH to mitochondrial CYP11B isozymes is facilitated by two flavoproteins, adrenodoxin reductase and adrenodoxin, also called ferredoxin reductase and ferredoxin. Both the ZF and ZR express CYP11B1 (Nishimoto *et al.* 2010, Gomez-Sanchez *et al.* 2014) which catalyses the 11β-hydroxylation (Figure 2.5; pink) of 11-deoxycortisol and DOC to cortisol and CORT, respectively, and A4 to 11OHA4 (Figure 2.7) (Swart *et al.* 2013, Van Rooyen *et al.* 2018). Although T is not a major adrenal product, it can also be hydroxylated to 11OHT (Chang *et al.* 1963) with reports of low levels detected in adrenal vein samples (Rege *et al.* 2013). In 2013, Swart *et al.* showed that *in vitro* CYP11B1 catalysed the conversion of A4 and T to their 11-hydroxylated products more readily compared to CYP11B2 (Swart *et al.* 2013). The expression of CYP11B1 is regulated by the HPA negative feedback loop stimulated by low glucocorticoid levels resulting in an increase in ACTH release, which in turn stimulates cAMP-dependent signalling pathways inducing gene transcription (Wang *et al.* 2000, Miller and Auchus 2011). In 2016, Imamichi *et al.* reported CYP11B1 mRNA transcripts and protein expression in human Leydig cells and ovarian theca cells which mediate the production of 11KT (Imamichi *et al.* 2016). Since the steroidogenic enzymes required for *de novo* steroidogenesis are expressed in the gonads, potential alternative sites for C11-oxy C_{21} steroid biosynthesis are therefore possible. 11β-hydroxylase activity has also been detected in testicular tumours (Savard *et al.* 1960) having further clinical implications with the potential production of C11-oxy active androgens. In addition, CYP11B1, which is primarily an enzyme catalysing the 11β-hydroxylase reaction, has been reported to exhibit low 18-hydroxylase activity with levels of 18OH-CORT detected in the serum of primary aldosteronism patients (Auchus *et al.* 2007) as well as *in vitro* CYP11B1 conversion assays yielding 18-hydroxy-DOC (18OH-DOC) (Fisher *et al.* 2001) —an activity more specific to CYP11B2. In 1996, Böttner *et al.* showed that by mutating the amino acid residues, L301, E302, A320, the CYP11B2 catalytic activity was decreased and resembles the 11β-hydroxylase activity of CYP11B1 (Böttneri *et al.* 1996).

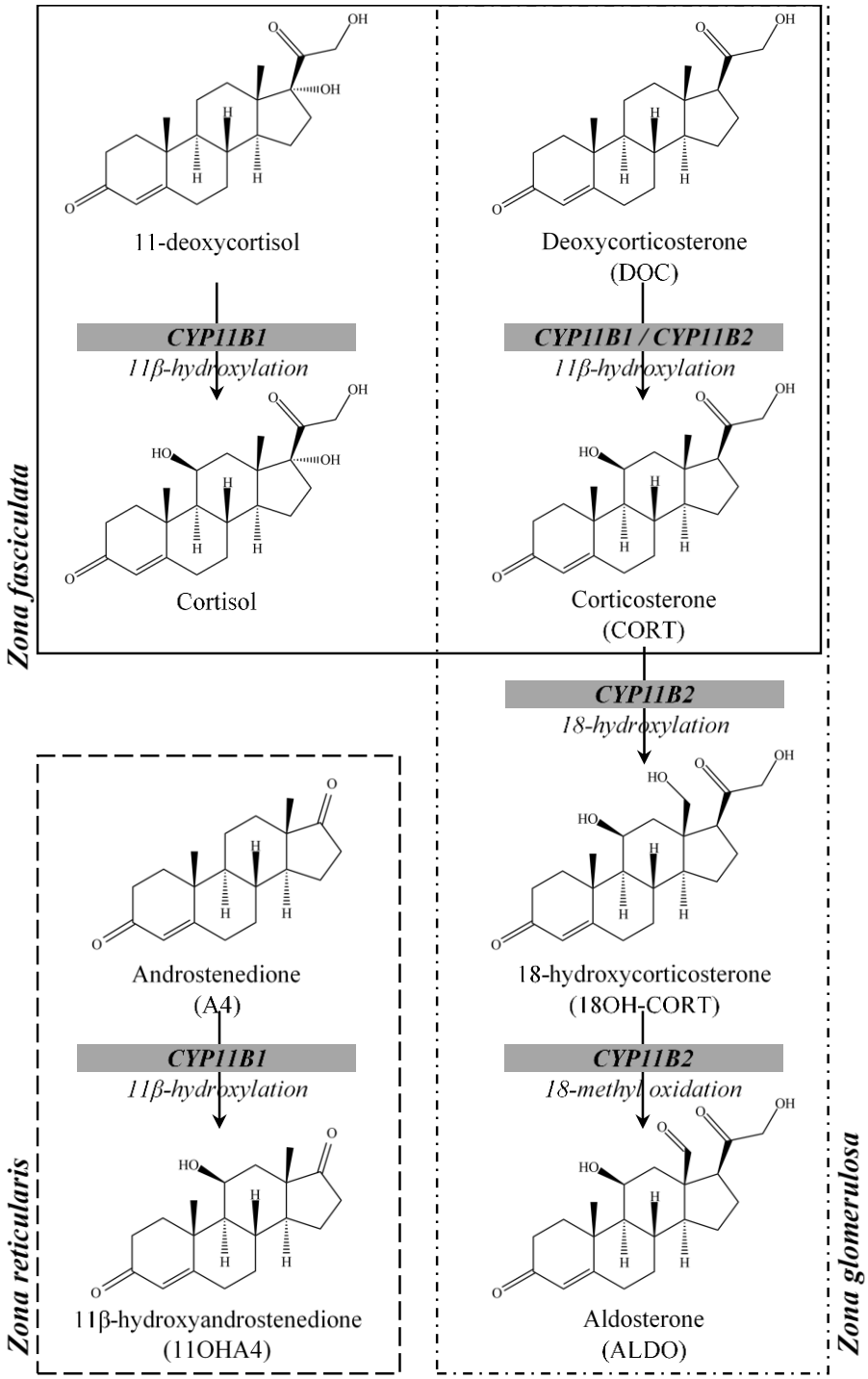

Figure 2. 7 CYP11B1 and CYP11B2 catalysed reactions yielding ALDO, cortisol and A4. CYP11B1 catalyses the conversion of 11-deoxycortisol and DOC to cortisol and CORT in the ZF (box, unbroken line) and A4 to 11OHA4 in the ZR (box, dashed line), and CYP11B2 converts DOC to ALDO in a three-step reaction yielding CORT and 18OH-CORT as intermediates (box, dot-dash line).

CYP11B2 is expressed in the ZG catalysing the 11β-hydroxylase, 18-hydroxylation and subsequent 18-methyl oxidation of DOC yielding the potent mineralocorticoid, ALDO (Figure 2.7) (Curnow *et al.* 1991, Kawamoto *et al.* 1992). The ability of CYP11B2 to convert the final oxidation reaction yielding ALDO was attributed to an internal hydrogen bond between C18 and C20 which stabilises 18OH-CORT in the active site and subsequently facilitates the oxidation reaction (Roumen *et al.* 2007). In addition, a similar docking study showed that CYP11B2 catalysed both the 11- and 18-hydroxylation of DOC with a preference for the latter yielding 18OH-DOC. This was further supported by gas chromatography-mass spectrometry (GC-MS) analysis of the *in vitro* conversion of DOC by CYP11B2 (Hobler *et al.* 2012). Nevertheless, both products are readily converted to 18OH-CORT *in vitro* (Fisher *et al.* 2001). Furthermore, *in silico* and *in vitro* studies showed, in addition to the 11β- and 18-hydroxylase activity, 19-hydroxylase activity by CYP11B2 yielding 19-hydroxylated DOC and CORT metabolites (Hobler *et al.* 2012).

As previously mentioned, the substrate specificity also extends to A4 and T. In addition, our group has also shown P4, 17OHP4 and 16OHP4 as substrates for both isozymes yielding 11OHP4, 21dF and 4-pregnen-11β,16α-diol, 3,20-dione (Barnard *et al.* 2017, Van Rooyen *et al.* 2017). As mentioned the expression of CYP11B2 is mainly regulated by the RAAS system –AngII and potassium induce an increase in intracellular calcium levels which activate phosphorylation cascades such as the calcium/calmodulin-dependent protein kinase which subsequently stimulates transcription factors (Nurr1 and NGF1B) resulting in gene transcription (Hanukoglu 1992, Clyne *et al.* 1997, Bassett *et al.* 2004, Szekeres *et al.* 2009). A recent study provided *in vitro* and *in vivo* evidence which showed human chorionic gonadotropin (hCG) upregulates CYP11B transcripts in eel testis as well as increasing testicular 11KT production (Ozaki *et al.* 2019). Abnormal expression patterns have also been observed in clinical conditions with Biancolella *et al.* (2012) reporting an increase in CYP11B2 expression in LNCaP cells treated with the SRD5A inhibitor, dutasteride (Biancolella *et al.* 2007). Testicular adrenal rest tumours (TART) express both adrenal and Leydig cell-specific steroidogenic enzymes which include CYP11B1 and CYP11B2 with expression levels similar to levels in the adrenal (Claahsen-van der Grinten *et al.* 2007, Lottrup *et al.* 2015, Smeets *et al.* 2015). Furthermore, Hartmann *et al.* (2019) has reported that the *in vivo* metabolism of spironolactone, an exogenous compound used in the treatment of hypertension, yields 11β- and 18-hydroxy derivatives, suggestive of CYP11B activity. Therefore, besides potential extra-adrenal 11β-hydroxylation, the adrenal re-uptake of exogenous and/or endogenous

compounds in circulation is plausible and these can, as such, be metabolised by enzymes expressed in the adrenal (Hartmann *et al.* 2019). CYP11B2 was previously implicated in the 11β- and 18-hydroxylation of metadienone, an anabolic steroid used for doping (Parr *et al.* 2012). Although cortisol and ALDO are generally regarded as the adrenal end-products of CYP11B isozyme activity in the glucocorticoid and the mineralocorticoid biosynthesis pathways, cortisol and the mineralocorticoid intermediate, CORT, serve as substrates for 11βHSD2 and, together with 11OHA4 and 11OHT, lead to the production of the C11-keto derivatives in the adrenal, albeit at low levels. While both 11βHSD isozymes, mRNA and/or protein, are expressed at very low levels in the adrenal (Mazzocchi *et al.* 1998, Ricketts *et al.* 1998, Mune *et al.* 2003) conversion of the C11-oxy steroids take place primarily in peripheral tissue such as the kidney, liver, testis, ovary, adipose tissue and skin (Smith *et al.* 1996, Bujalska *et al.* 1997, Ricketts *et al.* 1998, Tomlinson *et al.* 2004, Imamichi *et al.* 2016). 11βHSD isozymes play a crucial role in the metabolism of C11-oxy C_{19} and C11-oxy C_{21} steroids. In addition, these enzymes are important in the pre-receptor regulation of corticosteroid and C11-oxy C_{19} steroid hormone action, and may potentially also regulate that of C11-oxy C_{21} steroid hormone action. The 11βHSD isozymes are discussed in detail below.

2.3.4 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) and type 2 (11βHSD2)

Microsomal 11βHSD isozymes are important intracellular pre-receptor regulators of glucocorticoid and androgen action and catalyse the interconversion of 11-hydroxy and 11-keto steroids (Figure 2.5; red). Both isozymes are bound to the endoplasmic reticulum membrane facing either the cytoplasm (11βHSD2) or lumen (11βHSD1) with their catalytic activity determined by cofactor availability (White *et al.* 1997, Maser *et al.* 2002).

11βHSD2 is a dehydrogenase enzyme which uses the cytoplasmic cofactor, nicotinamide adenine dinucleotide (NAD^+), to catalyse the oxidation of 11-hydroxy steroids to 11-keto steroids which include the conversion of cortisol to cortisone and CORT to 11-DHC, occurring at low substrate levels and resulting in inactive glucocorticoids (Figure 2.8) (Rusvai and Naray-Fejes-Toth 1993). This activity is of biological importance due to the mineralocorticoid receptor's (MR) high affinity for both glucocorticoids and mineralocorticoids (Arriza *et al.* 1987, Funder *et al.* 1988). Mineralocorticoid levels (ALDO, ~83-278 pM) (Tan *et al.* 2020) in circulation are about 100 to

1000-fold lower (Rusvai and Naray-Fejes-Toth 1993) than cortisol (~ 300 nM) with the latter also increasing 2-fold during pregnancy (Hill *et al.* 2019). Thus, the MR and 11βHSD2 are often co-expressed in mineralocorticoid-responsive tissue in order to inactivate cortisol and allow the mineralocorticoid ligand to bind and activate the MR.

11βHSD1 is both a reductase and dehydrogenase with the reaction dependent on the NADPH/$NADP^+$ cofactor ratio as well as substrate concentration levels —11βHSD2 exhibits a relatively high affinity for steroids but 11βHSD1 does not and thus requires high substrate levels to facilitate the conversion (White *et al.* 1997). 11βHSD1 is often expressed in glucocorticoid target tissue where it can catalyse the activation of glucocorticoids. In an *in vitro* system, 11βHSD1 favours the oxidative reaction yielding the keto-derivative, however, the reductase activity is dominant *in vivo* with a lower apparent Km for cortisone than for cortisol, thus, yielding potent glucocorticoid metabolites (Chapman *et al.* 2013). This phenomenon is due to the interaction of H6PDH, also situated in the endoplasmic reticulum lumen, together with 11βHSD1, which regenerates $NADP^+$ preventing the $NADP^+$-facilitated inhibition of the reduction reaction (Atanasov *et al.* 2004, 2008, Bánhegyi *et al.* 2004). This finding was highlighted in a H6PDH knock-out study in murine macrophages showing decreased 11-oxoreduction and no oxidative activity (Marbet *et al.* 2018).

11βHSD1 catalyses the reduction of 11-keto steroids (cortisone to cortisol) while the oxidative reaction occurs at low NADPH/$NADP^+$ ratios (Figure 2.8). Placenta tissue expresses both isozymes and exhibits high $NADP^+$ levels which results in both isozymes contributing to the inactivation of glucocorticoids in order to protect the fetus (White *et al.* 1997).

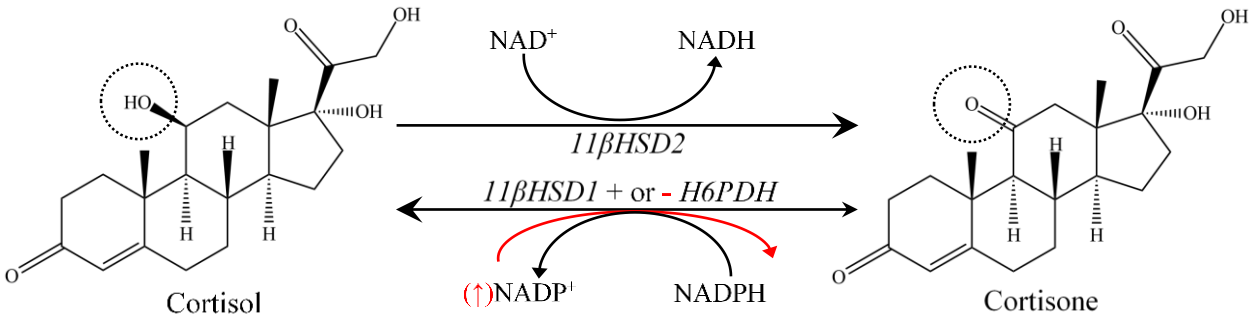

Figure 2. 8 Interconversion of cortisol and cortisone catalysed by the 11βHSD isozymes. 11βHSD2 utilises NAD^+ to oxidise cortisol to cortisone. 11βHSD1 is bidirectional and catalyses the oxidation of cortisol at low $NADPH/NADP^+$ levels but favours the reduction of cortisone to cortisol in vivo.

11βHSD isozymes also catalyse the interconversion of 11-hydroxy and 11-keto C_{19} steroids in the 11OHA4 pathway (Figure 2.3; green) (Swart *et al.* 2013). In contrast to the glucocorticoids being inactivated by 11βHSD2, the oxidation of C11-oxy C_{19} steroids result in increased androgenicity with the conversion of 11OHT and 11OHDHT to 11KT and 11KDHT resulting in potent androgens (Bloem *et al.* 2015). More recently, 11βHSD isozymes were shown to catalyse the interconversion of C11-oxy C_{21} steroids which include 11OHP4 and 11KP4 (Van Rooyen *et al.* 2018, Gent *et al.* 2019), and 21dF and 21dE (Barnard *et al.* 2017). 11βHSD1 expression has been detected in the liver, adipose tissue, luteinising granulosa cells, skeletal muscle and adrenal cortex with higher levels detected in the ZR and lower levels in the ZF (Tannin *et al.* 1991, Ricketts *et al.* 1998, Chapman *et al.* 2013) while 11βHSD2 is expressed in the gonads, adrenal, kidney, placenta, prostate and skin (Mune *et al.* 2003, Miller and Auchus 2011, Rege *et al.* 2013).

In the downstream metabolism of the aforementioned C11-oxy steroids, cortisol and cortisone also serve as substrates for SRD5A, yielding inactive metabolites (Nixon *et al.* 2012) while in the 11OHA4 pathway the 5α-reduction yields more potent androgens in the conversion of 11KT to 11KDHT and 11OHT to 11OHDHT (Bloem *et al.* 2013), similar to the conversion of T to the potent androgen, DHT.

2.3.5 5α-reductases (SRD5A)

Four SRD5A isozymes have been reported which include SRD5A1, SRD5A2, SRD5A3 and SRD5A2L2 but only SRD5A1 and SRD5A2 have been shown to catalyse the 5α-reduction reaction (Stiles and Russell 2010). SRD5A1 and SRD5A2 are microsomal enzymes which catalyse the irreversible NADPH-dependent reduction of C4-C5 double bond of steroids (Figure 2.5; orange) converting, for example, T to DHT (Russell and Wilson 1994). These enzymes are crucial in the biosynthesis of potent androgens in the conventional-, alternative-, 11OHA4- and backdoor pathway. SRD5A1 and SRD5A2 are expressed in numerous tissue with SRD5A2 the dominant isozyme in the healthy prostate, testis and genital skin (Bonnet *et al.* 1993, Russell and Wilson 1994, Marti, Galvan, *et al.* 2017) while SRD5A1 is the dominant isozyme in skin, liver (Thigpen *et al.* 1993) and ovary (Haning *et al.* 1996, Marti, Galvan, *et al.* 2017). SRD5A has been linked to many clinical conditions and diseases with increased expression of SRD5A1 is associated with PCa specifically CRPC (Bonkhoff *et al.* 1996, Titus *et al.* 2005, Stanbrough *et al.* 2006, Chang *et al.* 2011) while, in contrast, decreased levels have been reported in benign prostatic hyperplasia (BPH) (Bonnet *et al.* 1993). The role of SRD5A in producing potent androgens make them ideal targets in androgen deprivation therapies in which SRD5A inhibitors are administered preventing androgen activation. SRD5A activity was also reported to be elevated in PCOS (Jakimiuk *et al.* 1999, Chin *et al.* 2000, Vassiliadi *et al.* 2009, Torchen *et al.* 2016) and in 21OHD (Kamrath, Hartmann, *et al.* 2012).

While the SRD5A isozymes play a pivotal role in steroid activation, once converted to the 5α-reduced steroids, these are inactivated by HSD yielding C3-hydroxyl derivatives. However, the HSD isozymes are also capable of catalysing the reduction or oxidation of the keto and hydroxyl moieties at C17 and C20 yielding steroid hormones exhibiting various degrees of biological activity.

2.3.6 Hydroxysteroid dehydrogenases (HSD)

HSD enzymes can be classified as either short-chain dehydrogenase/reductase (SDR) enzymes which include 3βHSD, 11βHSD, 17βHSD1 and 17βHSD2, or as aldo-keto reductase (AKR) enzymes which include 3αHSD and 20α-HSD. These enzymes utilise either NAD(H) or NADP(H) to catalyse the reduction or oxidation of steroid compounds (Penning 1997). Although there are a

number of HSD isozymes capable of catalysing steroids, the focus will be placed on a selected number of enzymes pertinent to this study.

Four 3αHSD are found in humans which catalyse the reversible reduction of C3-, C17- and C20-keto steroids or the oxidation of 3α-, 3β-, 17β- and 20α-hydroxy steroids. These include 3αHSD type 1 (3αHSD1), 3αHSD type 2 (3αHSD2), 3αHSD type 3 (3αHSD3) and 3αHSD type 4 (3αHSD4). All four isoforms are capable of catalysing the interconversion of DHT and 3αAdiol, estrone to estradiol, P4 to 20α-hydroxyprogesterone (20αOHP4) to various degrees but the direction of the reaction is dependent on the cofactor availability (Penning *et al.* 2000). 3αHSD1, also called AKR1C4, is abundantly expressed in the liver and exhibits both 3α- and 20α-reductase activity resulting in the inactivation of DHT, P4 and DHP4 to 3αAdiol, 20αOHP4 and Allo, respectively (Dufort *et al.* 2001, Higaki *et al.* 2003). Although protein expression has been reported in normal adrenal tissue and in adrenocortical carcinomas, the levels were low (Marti, Malikova, *et al.* 2017). 3αHSD3, or AKR1C2, uses electrons from NADPH to catalyses the 3-keto reduction (Figure 2.5; green) of C_{19} steroids in the conventional and alternative pathways (DHT to 3αAdiol) yielding metabolites which are readily conjugated (Dufort *et al.* 2001, Rižner *et al.* 2003) and in the 11OHA4 pathway to yield inactive steroids which exhibit a lower affinity for the AR and are inefficiently conjugated (Figure 2.3). Studies have shown 3αHSD3 to catalyse the oxidation of 3αAdiol to DHT *in vitro* using both NAD^+ and $NADP^+$ as electron sources and favouring 3αAdiol. However, the oxidation reaction is dependent on the NADPH:NAD^+ ratio, as high NADPH levels inhibited NAD^+-dependent oxidation of 3αAdiol (Rižner *et al.* 2003). In the backdoor pathway, 3αHSD3 catalyses the biosynthesis of 3αAdiol from AST which is subsequently converted to DHT (Auchus 2004). The expression of 3αHSD3 in target tissue therefore serves as a regulator of androgen activation (Penning *et al.* 1996, Biswas and Russell 1997, Miller and Auchus 2011). Expression levels of 3αHSD3 are high in mammary glands and in the prostate (Penning *et al.* 2000) with little variation observed between diseased or hyperplastic prostate tissue and healthy prostate tissue (Penning *et al.* 2007). However, elevated expression levels have been reported in endometrial cancer cell lines (Sinreih *et al.* 2015). Dufort *et al.* (2001) had previously showed a significant reduction of P4 at C20 *in vitro* (Dufort *et al.* 2001). 3αHSD4 **(**AKR1C1 or 20αHSD) also catalyses the reduction of P4 to yield the inactive 20αOHP4 with negligible oxidase activity mediating P4-induced activity. Although the 20α-reduction is the preferred reaction, 3αHSD4 yields both 3α/β- and 20β-hydroxy steroids (Penning and Byrns 2009). While 3αHSD4 can use

both NADH and NADPH *in vitro*, a higher affinity towards NADPH has been reported (Zhang *et al.* 2000). The enzyme is widely expressed with mRNA levels detected in the liver, mammary gland, placenta, brain, testis, prostate and uterus (Zhang *et al.* 2000) as well in the ovary (Martel *et al.* 1992).

Although these enzymes' catalytic capabilities overlap, their tissue distribution differs. 3αHSD1, being specific to the liver, produces steroid metabolites which serve as substrates for conjugation. However, a reduced metabolite such as 3αAdiol, can be released back into circulation to be transported to other tissue such as the prostate where it can be reactivated yielding DHT. 3αHSD3 is widely expressed in tissue which includes the prostate (Penning *et al.* 2000), adrenal cortex, skin and brain —in the latter 3αHSD3 converts DHP4 to the neuroactive Allo (Concas *et al.* 1998). 3αHSD2 is generally referred to as AKR1C3 or 17βHSD5 and is therefore discussed with the 17βHSD isozyme below.

To date, fourteen 17βHSD have been identified in humans and are all classified as SRD enzymes with the exception of 17βHSD5 (Moeller and Adamski 2009, Tsachaki and Odermatt 2019). These isozymes catalyse the NAD(P)H-dependent reduction and $NAD(P)^+$-dependent oxidation of steroid hormones of which the reductive isozymes, 17βHSD type 1 (17βHSD1), 17βHSD3 and 17βHSD type 5 (17βHSD5), and dehydrogenase, 17βHSD2, are more prevalent in aforementioned steroidogenic pathways. 17βHSD isoforms favour either the reductive or oxidative reaction, depending on cofactor levels and substrate availability (Mindnich *et al.* 2005). This discussion will focus on selected enzymes relevant to this study.

17βHSD1 is a cytosolic enzyme (Tsachaki *et al.* 2015), also known as estrogenic 17βHSD, which catalyses the NADPH-dependent reduction of C_{18} steroids such as estrone to estradiol while exhibiting a lower affinity for C_{19} steroids as in the conversion of DHEA to A5. The enzyme is mainly expressed in the placenta, breast and granulosa cells of the ovary with lower levels detected in endometrium (Luu The *et al.* 1989, Martel *et al.* 1992, Andersson *et al.* 1995, Ann *et al.* 1996, Peltoketo *et al.* 1999). An increase in expression has been reported in estrogen-sensitive carcinomas which include the endometrium and breast cancers (Gunnarsson *et al.* 2001, Oduwole *et al.* 2004). Similarly, cytosolic 17βHSD14 is reported to regulate intracellular active steroid levels by inactivating estrogens in tissues such as the placenta, breast and brain but uses NAD^+ to facilitate the conversion (Lukacik *et al.* 2007, Tsachaki and Odermatt 2019). 17βHSD14 also

converts A5 and T to DHEA to A4, respectively (Lukacik *et al.* 2007). Other isozymes such as 17βHSD7 and 17βHSD12 have been reported to be less efficient in reducing estrone to estradiol in transfected HEK293 cells than 17βHSD1 (Laplante *et al.* 2009).

17βHSD2 utilises NAD^+ for the oxidation of the 17β-hydroxy group of androgens and estrogens such as DHT and estradiol to the inactive 5αDione and estrone metabolites, respectively, while also oxidising 20αOHP4 at C20 to yield P4, a potent progesterone (Miettinen *et al.* 1996). Microsomal 17βHSD2 is a membrane-bound enzyme with the active site orientated towards the cytosol (Tsachaki and Odermatt 2019). The enzyme catalyses the 17β-oxidation of C_{19} and C11-oxy C_{19} steroids in the androgen pathways —T to A4, DHT to 5αDione, 3αAdiol to AST and their respective 11-hydroxy and 11-keto derivatives as well as A5 to DHEA (Wu *et al.* 1993, Elo *et al.* 1996). 17βHSD2 expression has been reported in the liver, placenta and small intestine together with low levels being expressed in the prostate and kidney while expression in the testis and ovary is negligible (Andersson *et al.* 1995, Moghrabi *et al.* 1997, Penning 1997, Peltoketo *et al.* 1999). 17βHSD4 is functionally similar to 17βHSD2, mediating the NAD^+-dependent peroxisomal β-oxidation of androgens and estrogens as well as that of fatty acids (Adamski *et al.* 1995, Penning 1997, Breitling *et al.* 2001).

17βHSD3 and 17βHSD5 are mainly 17-keto-steroid reductases which utilise electrons from NADPH and catalyse the reverse reaction of type 2 yielding androgens, some of which can activate the AR (Geissler *et al.* 1994, Dufort *et al.* 1999). In addition, type 5 can also yield 3α/β-hydroxy steroids (Steckelbroeck *et al.* 2004). 17βHSD3 is bound to the endoplasmic reticulum with the active site protruding towards the cytosol. It has been hypothesised that the activities of 17βHSD3 and 11βHSD1 are coupled by means of cofactors in which the $NADP^+$ generated from the 17βHSD3 reaction would drive the oxidative reaction of 11βHSD1 yielding inactive glucocorticoids (Hu *et al.* 2008). However, this was disproven *in vitro* by showing that 17βHSD3 is dependent in cytosolic glucose-6-phosphate dehydrogenase (G6PDH) and not H6PDH (Legeza *et al.* 2013). While 17βHSD5 has been shown to reside in the cytosol, the enzyme was shown to uniquely translocate with the AR into the nucleus upon ligand activation (Yepuru *et al.* 2013).

Both 17βHSD3 and 17βHSD5 can convert A4 to T, 5αDione to DHT and AST to 3αAdiol (Dufort *et al.* 1999, Mindnich *et al.* 2005) as well as catalysing the conversion of their C11-oxy derivatives to the respective products. 17βHSD3 is mainly expressed in the Leydig cells (Andersson *et al.*

1995, 1996) contributing to the biosynthesis of T (Geissler *et al.* 1994) while 17βHSD5 expression levels are higher in prostate tissue (Martel *et al.* 1992, Lin *et al.* 1997, Penning and Byrns 2009). In addition, 17βHSD5 is expressed in the theca cells of ovary (Pelletier *et al.* 1999) liver and mammary gland and reduces estrone to estradiol and P4 to 20αOHP4 (Penning *et al.* 2000, Penning and Byrns 2009). Higher 17βHSD5 activity *vs.* 17βHSD2 ensures adequate levels of potent androgens in the prostate. Barnard *et al.* (2018) reported the preference of 17βHSD5 for the C11-keto C_{19} steroids which included 11KA4, 11K-5αDione and 11KAST compared to C_{19} steroids (A4, 11KDHT and AST) with a 8 to 24-fold higher catalytic efficiency reported for the C11-oxy compounds (Barnard *et al.* 2018). 17βHSD5 is implicated in the progression of PCa and PCOS due to its role in the production of DHT and 11KT via the conventional-, alternative- and backdoor pathway (Auchus 2004) and the 11OHA4 pathway (Bloem *et al.* 2015, Barnard *et al.* 2018). Expression of 17βHSD5 is elevated in PCOS patients associated with hyperandrogenism with levels exceeding that of 17βHSD3 and 17βHSD6 (Penning 2019). Lastly, 17βHSD6 has been reported to reduce 3αAdiol to AST, DHT to 3αAdiol as well as catalyse the oxidative reaction of 3αAdiol to DHT (Mohler *et al.* 2011). Interestingly to note, the mitochondrial 17βHSD10 has been suggested to play a role in the backdoor pathway catalysing the 3αAdiol oxidation yielding DHT (Penning 2010). Certain 17βHSD enzymes are expressed in lipid droplets and include 17βHSD11 and 17βHSD13 (Tsachaki and Odermatt 2019) with 17βHSD11 reported to exhibit 17β-oxidation activity towards estradiol and 3αAdiol yielding estrone and AST, respectively (Brereton *et al.* 2001).

Lastly, 17βHSD6, 7, 9 and 12 are bound to the endoplasmic reticulum membrane with the active sites facing the lumen (Tsachaki and Odermatt 2019). 17βHSD6 and 17βHSD9, sometimes called retinol dehydrogenase (RODH) and 11-cis-RODH, respectively, are dehydrogenases which can catalyse the 17β-oxidation of estradiol and androgen as well as the 3α-oxidation of androgens. The latter is a crucial reaction in the backdoor pathway in which the inactive 3αAdiol is converted to the biologically active androgens, DHT. However, RL-HSD exhibited higher activity for this conversion and has thus been implicated not only in the activation of androgens but also in the backdoor pathway catalysing the ultimate conversion of 3αAdiol to DHT by oxidising the 3α-hydroxy to a keto group (Figure 2.5; green) (Biswas and Russell 1997, Auchus 2004, Bauman, Steckelbroeck, Williams, *et al.* 2006). In addition, the enzyme converts the inactive androgens — AST, 3αAdiol and their respective C11-oxy derivative, to 5αDione, DHT and the C11-oxy

derivatives, respectively, in the conventional-, alternative- and 11OHA4 pathway and also reduces 3-keto steroids to the 3β-derivatives. In the absence of this oxidation step, C_{19} and C11-oxy C_{19} steroids remain inactive and present substrates for conjugation and subsequently secretion from the cell. RL-HSD is often co-expressed with the AR in prostatic stromal cells creating an environment in which DHT, biosynthesised from 3αAdiol, can transactivate the AR (Biswas and Russell 1997, Bauman, Steckelbroeck, Peehl, *et al.* 2006). Expression of the enzyme has also been reported in BPH cells (Bauman, Steckelbroeck, Peehl, *et al.* 2006, Penning *et al.* 2007) as well as elevated transcript levels being detected in PCOS (Wood *et al.* 2003). In addition, 17βHSD10, RODH4, RDH5, and DHRS9 have also been shown to catalyse the activation of 3αAdiol yielding DHT (Bauman, Steckelbroeck, Williams, *et al.* 2006, Mohler *et al.* 2011).

It is evident that the tissue-specific expression and levels of the various enzymes, together with the substrate and cofactor availability, determine the metabolic end-product which can subsequently mediate a physiological process by binding to a steroid nuclear receptor.

2.4 Bioavailability

Steroid receptors are often co-expressed with HSD which act as pre-receptor regulators —yielding either inactive steroid metabolites or hormones which can bind a receptor and induce a response such as transactivation, transrepression or act as a competitor. In addition, the potency of a steroid can be attenuated temporarily or indefinitely by binding to steroid hormone-binding globulins and/or sulfation, or irreversibly by glucuronidation which will be discussed in the following section.

2.4.1 Uridine-diphosphate glucuronosyltransferases (UGT)

UGT enzymes conjugate steroids irreversibly, most often those containing a 3α- and/or 17β-hydroxyl group such as DHT and 3αAdiol, with a glucuronic acid moiety derived from uridine 5'-diphosphoglucuronic acid (Figure 2.9) in order to increase the steroid's hydrophilicity for urinary excretion (Tukey and Strassburg 2000). Glucuronidated steroids are unable to bind a receptor and elicit a biological response. Various UGT enzymes exist in humans and play an important role in the metabolism of steroids as well as xenobiotic compounds and are classified into three subfamilies, UGT1A, UGT2A and UGT2B —the latter being of importance in steroid

metabolism. UGT2B expression have been reported in tissue which include the liver, kidney, mammary gland, lung and prostate gland. UGT2B type 7, type 15, type 17 and type 28 catalyse the glucuronidation of androgens which include DHT, AST, T and 3αAdiol (Bélanger *et al.* 2003); UGT2B15 favours conjugation at 17β position of C_{19} steroids such as T and DHT (Pelletier *et al.* 1992) while the other UGT2Bs can catalyse glucuronidation at both 3α- and 17β-hydroxyl groups. UGT2B17 readily conjugates AST and showed the lowest affinity for T (DHT > 3αAdiol >T) (Beaulieu *et al.* 1996).

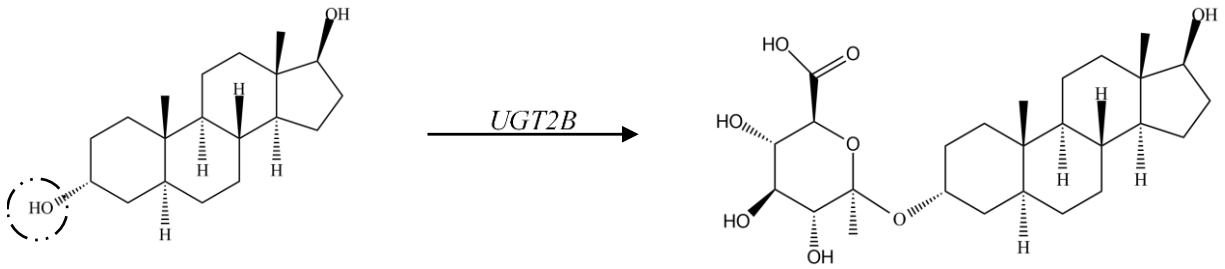

Figure 2. 9 Schematic representation of the glucuronidation of 3αAdiol at C3.

Interestingly, circulatory AST-glucuronide levels are higher compared to unconjugated AST levels (Bélanger *et al.* 1991). Although glucuronidation is most prominent in the liver and kidney (Hartmann *et al.* 2019), glucuronidation activity has also been detected in numerous other tissue which include the prostate and PCa tissue (Beaulieu *et al.* 1996, Ric Lévesque *et al.* 2001, Bélanger *et al.* 2003, Du Toit *et al.* 2017), testis, ovaries, breast, skin, brain as well as the adrenal gland (Beaulieu *et al.* 1996, Ric Lévesque *et al.* 2001). LNCaP cells express UGT2B10 and UGT2B15 (Bélanger *et al.* 1991, 1995) while breast cancer cell models which include MCF-7 and ZR-75-1 were also shown to express UGT2B (Adams *et al.* 1989). More recently, Du Toit *et al.* showed the inefficient glucuronidation of the C11-oxy C_{19} steroids suggesting that the C11-oxy moiety hinders the conjugation reaction allowing C11-oxy C_{19} steroids to remain active longer compared to C_{19} steroids (Du Toit *et al.* 2017, Du Toit and Swart 2018). However, the mechanism leading to the inefficient conjugation and the status of C11-oxy C_{21} steroid glucuronidation remain largely unexplored with only 11KP4 having been reported in a glucuronidated form in the plasma of BPH patients (Du Toit and Swart 2020). Another mechanism of steroid inactivation is sulfation but, unlike glucuronidation, sulfation is reversible and can also serve as a steroid reservoir.

2.4.2 Sulfotransferases (SULT) and sulfatases (STS)

Sulfation of steroids play an important role in the body, particularly during pregnancy when the fetus requires protection from maternal steroid levels (Geyer *et al.* 2016). Sulfate conjugation is catalysed by sulfotransferases (SULT) enzymes. They utilize 3-phospo-adenosine-5'-phosphosulfate (PAPS) as a sulfate donor which is synthesized from ATP and inorganic sulfate. The latter reaction is catalysed by 3-phospo-adenosine-5'-phosphosulfate synthases (PAPSS) with two isoforms identified in humans PAPSS1 and PAPSS2.

A sulfate group (SO_4^{-3}) is transported into the cells and metabolised in a two-step reaction catalysed by PAPSS to yield an active sulfate in the form of PAPS. Cytosolic SULT subsequently utilises PAPS for sulfuryl transfer to the steroid hydroxyl group, at either C3 or C17, with the sulfate moiety rendering it temporarily inactive and water-soluble.

Thirteen SULT enzymes have been identified in humans which catalyse the sulfation of various compounds ranging from steroids to peptides. Four of the SULT family enzymes, which include SULT1E1, SULT2A1, SULT2B1a and SULT2B1b, catalyse the sulfation of steroids and/or cholesterol. SULT2B1a and SULT2B1b are more restricted with regards to substrate specificity where SULT2B1b catalyses the sulfation of mainly cholesterol and to a lesser degree P5 and DHEA, while SULT2B1a sulfonates P5 and DHEA and not cholesterol (Fuda *et al.* 2002, Strott 2002). Higher levels of SULT2B1b was detected in the prostate compared to SULT2B1a. Both isozymes are, however, decreased in PCa resulting in higher levels of steroid precursors, cholesterol and P5 (Seo *et al.* 2013). In addition, SULT2B1b expression in PCa cell models had an inhibitory effect on AR activity and expression and subsequently on cell proliferation (Vickman *et al.* 2016) which suggest that the lower expression levels reported in PCa (Seo *et al.* 2013) would contribute to disease progression

SULT2A1 is the main steroid SULT enzyme which mediates the sulfuryl transfer to 3α- (AST), 3β- (P5, 17OHP5, DHEA and A5) and 17β-hydroxyl (T, DHT and estradiol) moieties of steroids as well as the phenolic hydroxyl group of estrone and estradiol (Radominska *et al.* 1990, Javitt *et al.* 2001, Rainey and Nakamura 2008, Coughtrie 2016). During childhood, DHEA is the main substrate for SULT2A1 resulting in high circulating levels of DHEAS, > 5 µM, which far exceed that of DHEA levels, > 15 nM (Rege, Garber, *et al.* 2019) which, together with A5-sulfate, steadily decline with age (Orentreich *et al.* 1984, Bélanger *et al.* 1994). SULT2A1 is widely expressed in

tissue such as the ZR of the adrenal (Endoh *et al.* 1996, Rainey and Nakamura 2008), liver, kidney (Sheng and Duffel 2003) and granulosa cells (Lerner *et al.* 2019). Elevated DHEAS have been reported in PCOS while DHEAS was decreased in 21OHD together with P5- elevated sulfate (Turcu *et al.* 2015, 2016, Rege, Turcu, *et al.* 2019). SULT1E1 has a high affinity for estrogens, particularly estradiol (Zhang *et al.* 1998) but can also conjugate DHEA (Hempel *et al.* 2000). Sulfated steroids in circulation are unable to cross the lipophilic cellular membrane and are thus transported into the cell by anion-transporters such as sodium-dependent organic anion transporters where they are subsequently desulfated by steroid sulfatases (STS) such as arylsulfatase C. STS remove the sulfate group and restore the steroid hydroxy group rendering the steroid free for subsequent metabolism or receptor interactions. These enzymes are embedded in the endoplasmic reticulum membrane facing the lumen (Thomas and Potter 2013) and are widely expressed in tissue which include, amongst others, male and female reproductive tissue, adrenal, kidney, and skin (Warren and French 1965, Reed *et al.* 2005) STS mRNA levels were reported to exceeded that of SULT1E1 in cancerous cell models such as MCF-7, Ishikawa, JEG-3 and HepG2 (Šmuc and Rižner 2009).

The products of glucuronidation and sulfation are more water-soluble which allows them to be transported and/or excreted in hydrophilic matrices such as blood and urine. While chemical alterations to the steroid structure are required for steroid-sulfate biosynthesis, steroids can also be transported in the circulation bound to steroid binding proteins which do not required major transformations.

2.4.3 Steroid hormone-binding protein (SHBP)

The hydrophobic nature of steroids requires their transport in circulation in the bound form which necessitates a glycoprotein chaperone. Steroid hormone-binding proteins such as sex hormone-binding globulin (SHBG), androgen-binding protein, corticosteroid-binding globulin (CBG) and albumin temporarily bind and inhibit steroid activity, transporting the steroid to the target tissue where they are subsequently released in order to diffuse into the tissue (Barahona *et al.* 1980). However, the mechanism resulting in the release and/or transport of the steroid into the target tissue has, to date, not been fully elucidated. Most of the steroids in circulation are bound by these proteins with merely 1-3 % of circulating steroids being detected as unbound steroids (Dunn *et al.*

1981). Although chaperone proteins were initially believed to originate in the liver and to be limited to circulation, studies have since reported extrahepatic origins such as gonadal tissue, breast and prostate (Hagenäs *et al.* 1975, Vigersky *et al.* 1976, Barahona *et al.* 1980, Kahn *et al.* 2002, Fortunati *et al.* 2010, Ma *et al.* 2013) and intracellular localisation and membrane interaction have been reported (Hryb *et al.* 1985, Nakhla *et al.* 1990, Kahn *et al.* 2002, Caldwell and Jirikowski 2014). Thus, in addition to the chaperone function an increasing number of studies are reporting an intracellular regulatory role of SHBG with regards to steroid activity (Li *et al.* 2016).

These proteins exhibit different affinities towards different steroids; SHBG binds T, DHT and 3αAdiol with a high affinity while exhibiting a lower affinity towards estradiol (Dunn *et al.* 1981). CBG has a high affinity for glucocorticoids and P4 (Mickelson *et al.* 1981). Albumin is abundant in circulation, binding most of the steroids, however, with a low affinity and specificity. It has been proposed that these steroids would also contribute to bioactivity due to the fast dissociation of steroids of which T is an example and that chaperone bound steroids serve as a reservoir preventing steroids from being excreted (Manni *et al.* 1985, Hammond 2011). In PCOS patients, SHBG levels were decreased resulting in higher levels of free circulating androgens which contribute to androgen-induced symptoms (Kopelman *et al.* 1980, Pugeat *et al.* 2010, Wang *et al.* 2019). In contrast, elevated expression levels in PCa were associated with disease progression (Ma *et al.* 2013). To date, the affinity of SHBP towards the C11-oxy steroids has not been investigated.

The presence of steroid binding proteins adds another layer of complexity to the manner in which steroids exert an effect. Variables such as substrate and cofactor availability and the enzyme expression profile determine the biosynthesis of end-products, however, the bioactivity of the steroids is further influenced by whether the steroid is free or conjugated or protein bound. Once free, the steroid can elicit a physiological response by binding a steroid receptor resulting in agonist or antagonist activity and the subsequent modulation of target gene expression.

2.5 Bioactivity

Classical nuclear receptors, which include the AR, progesterone- (PR), glucocorticoid- (GR), MR and estrogen receptors (ER), are ligand-induced transcription factors which mediate steroid signalling. Generally, the steroid ligand binds the receptor which induces a conformational change resulting in the release of corepressors and the association of coactivators which in turn activate

gene transcription (Aranda and Pascual 2001, Olefsky 2001). The diverse processes which maintain physiological homeostasis are dependent on the regulated expression of steroid receptors in target tissue and access to potent ligands. In this study, we focused on the PR and AR due to the association of C11-oxy steroids with tissue expressing these receptors and/or their association with androgen-dependent diseases and disorders.

The unliganded receptors generally reside in the cytoplasm bound to chaperone proteins such as the heat shock proteins (Hsp90 or Hsp56) which upon ligand binding dissociates from the AR or PR allowing dimerization. After phosphorylation, the receptor-ligand complex translocates into the nucleus and binds the DNA hormone response element resulting in transcriptional activity or repression (Aranda and Pascual 2001). Although the abovementioned is the general conventional model, studies have reported unbound receptor localisation in the nucleus, lack of dimerization and other signalling pathways (Aranda and Pascual 2001, Jacobsen *et al.* 2005, Balaguer *et al.* 2019, Ma and Nelson 2019).

T and DHT are the classical potent androgens which bind and activate the AR resulting in the induction of biological processes crucial in male reproductive physiology. Although 11OHA4 does not exhibit androgenic activity, studies have shown transactivation of the human AR by various C11-oxy C_{19} steroid metabolites such as 11KT and 11KDHT, which exhibit agonist activity similar to T and DHT (Yazawa *et al.* 2008, Bloem *et al.* 2015). The AR is abundant in the prostate and is co-expressed with RL-HSD, the enzyme which can re-activate 3αAdiol yielding DHT, in prostatic stroma cells. In addition, a mutated AR was identified in CRPC which not only exhibited similar binding affinities towards C_{19} steroids compared to the wild-type AR but was also transactivated by DHT and P4 (Culig *et al.* 1993, Cai *et al.* 2011), thus, P4 can also contribute to the progression of the disease. In females, the AR is expressed in follicular theca and granulosa cells and mediate processes important in ovulation and subsequently influence fertility (Walters *et al.* 2008). Hyperandrogenism and elevated AR expression in the ovary and endometrium of PCOS patients have also been linked to infertility in these subjects (Apparao *et al.* 2002, Catteau-Jonard *et al.* 2008).

The PR and AR are homologous in structure. The role of the PR in female reproduction has been studied extensively. Two isoforms have been described *viz.* isoform A (PR-A) and isoform B (PR-B) which are normally expressed at similar levels in healthy target tissue while disruption of

the PR-A:PR-B ratio being associated with disease (Graham and Clarke 1997). Although similar in structure and steroid binding, the isoforms differ in function with PR-A reported as a repressor of transcription and of PR-B activity while PR-B activates gene transcription. In addition, the isoforms are regulated independently from each other (Wen *et al.* 1994). The PR isoforms are expressed in various male and female tissue which include the testis, prostate, brain, uterus, ovary, breast and vagina (Wilson and McPhaul 1996, Graham and Clarke 1997, Yu *et al.* 2015, Chen *et al.* 2017). In addition, PR expression levels are elevated in PCa (Grindstad *et al.* 2015), however, controversial results have been reported (Yu *et al.* 2015). Although the androgenicity of C11-oxy C_{19} steroids have been investigated with regards to the wild-type AR, the activity of C11-oxy C_{21} steroids towards the AR and PR remains unexplored to date as are the C11-oxy C_{19} steroids towards the PR isoforms.

To date, a vast array of steroids has been reported to bind and/or activate the different nuclear receptors with some steroids able to bind more than one receptor. Numerous studies have investigated receptor expression levels in tissue, their possible ligands as well as quantifying steroid levels in circulation or in specific tissue to elucidate the steroidogenic environment and activity endeavouring to understand and possibly manipulate the mechanism underlying healthy and diseased cell proliferation, disease progression and other ligand-induced transcriptional activity (Aranda and Pascual 2001, Conzen 2008, Fuller 2015, Zhao *et al.* 2020). As previously mentioned, potent steroids such as DHT can elicit a response even at very low concentrations. It is therefore important to not assume that steroids which are detected at low levels are insignificant and not contributing to endocrinological homeostasis. In the last decade, mass spectrometry has become the golden standard for steroid identification and quantification and is able to accurately detect and measure steroids at pM concentrations. Consequently, due to technical advancements in analytical fields, steroid metabolites which circulate at very low levels are currently identified and reported more frequently.

2.6 Mass spectrometry

Chromatography-linked mass spectrometry is frequently used for the analysis of steroids in various matrices such as in cell culture medium, urine, serum and saliva. Mass spectrometry has many advantages over immunoassays which include improved calibration range, high-throughput, high

sensitivity and the ability to analyse numerous steroids simultaneously and is thus taking centre stage when it comes to small molecule analysis.

Currently, studies investigating steroids either report using GC-MS (Hill *et al.* 2019) or liquid chromatography-MS (LC-MS) methods with the latter becoming more prevalent (Quanson *et al.* 2016, Du Toit *et al.* 2018, Wudy *et al.* 2018, Häkkinen *et al.* 2019, Schiffer *et al.* 2019). Although GC-MS offers high resolution, sensitivity and is more cost efficient than LC-MS, the technique is laborious in terms of sample preparation as steroids need to be derivatised and deconjugated while also requiring highly specialised training. LC-MS, albeit at lower resolution, can detect and quantify a multitude of steroids extracted from a small sample volumes without the preparation required for GC-MS (Stanczyk and Clarke 2010). Furthermore, the use of supercritical fluid chromatography (SFC) have allowed for even greater resolution, sensitivity and selectivity which is ideal for analysis of compound such as steroids which closely resemble each other in structure. The use of SFC reduced the run time of analyses and improved the efficiency of the applied method. More studies are turning to ultra-high-performance LC-MS (UHPLC-MS) and ultra-performance convergence chromatography tandem mass spectrometry (UPC^2-MS/MS) as a means to determine steroid profiles and/or to identify potential clinical biomarkers. The importance of accurate quantification in generating comprehensive steroid profiles is frequently emphasised in various disciplines in which disease progression is monitored or in which biomarkers are identified and even in the professional sporting community where the athlete biological passport (ABP) is used to monitor the use of anabolic steroids. However, these methods are limited to only a few steroids which, due to the structural similarity of steroids, can be problematic due to steroid crosstalk. Analysis of steroid profiles focussing on a limited number of steroids would not account for a full complement of steroids present in biological samples. Therefore, the inclusion of all steroids relevant to a particular study should be considered while also compensating for potential steroid interferences in the accurate quantification of steroid hormones when establishing methods in order to eliminate false-positive steroid identification.

2.7 Summary

It is evident that various steroidogenic pathways and numerous enzymes can influence the metabolic fate of a steroid which emphasise the complexity of steroidogenesis. In the backdoor

pathway, DHT is biosynthesised from C_{21} steroids without A4 or T as intermediates which suggests that other C_{21} steroids, more specifically C11-oxy C_{21} steroids, are potential precursors to androgens. The importance of considering all possible metabolic pathways is highlighted by the steady rise in reports (Kamrath *et al.* 2017, Turcu *et al.* 2017, Hamer *et al.* 2018, Rege *et al.* 2018, Nanba *et al.* 2019, Rege, Garber, *et al.* 2019, Rege, Turcu, *et al.* 2019, Du Toit and Swart 2020) identifying 11OHA4 and its metabolites as well as the backdoor pathway activity in endocrine disorders and diseases, together with recently elucidated pathways associated with clinical conditions. Furthermore, the roles of steroidogenic enzymes are complex as the conversion by one enzyme can result in an inactive steroid, the same enzyme can catalyse the activation of another steroid thus exhibiting a regulatory role with regards to the biological response.

C11-oxy C_{19} steroids have been identified in various biological samples and exhibit androgenic activities similar to the classical androgens and are less efficiently eliminated from the body by glucuronidation —rendering them biologically active for longer periods. However, studies are only now identifying the C11-oxy C_{21} steroids *in vivo* and are limited to only one or two C11-oxy C_{21} hormones. This study therefore firstly aimed to investigate the biosynthesis of the C11-oxy C_{21} steroids by adrenal enzymes and their subsequent metabolism by steroidogenic enzymes, which will be addressed in the following chapter.

Chapter 3

Published manuscript: The *in vitro* metabolism of 11β-hydroxyprogesterone and 11-ketoprogesterone to 11-ketodihydrotestosterone in the backdoor pathway

3.1 Introduction

Human cytochrome P450 11β-hydroxylase (CYP11B1) and cytochrome P450 aldosterone synthase (CYP11B2) are adrenal enzymes which play a crucial role in the biosynthesis of mineralocorticoids and glucocorticoids. The enzymes catalyse the 11β-hydroxylation of androstenedione (A4) and testosterone (T) to 11β-hydroxyandrostenedione (11OHA4) and 11β-hydroxytestosterone (11OHT), respectively, which are metabolised in the 11OHA4 pathway yielding potent C11-oxy C_{19} steroids. In addition, CYP11B1 and CYP11B2 catalyse the 11β-hydroxylation of progesterone (P4) and 17α-hydroxyprogesterone (17OHP4) to 11β-hydroxyprogesterone (11OHP4) and 21-deoxycortisol (21dF), respectively (Schloms *et al.* 2012, Barnard *et al.* 2017).

The C11-oxy C_{21} steroids, 11OHP4 and 21dF are often reported in studies reporting increased backdoor pathway activity in adrenal disorders such as 21-hydroxylase deficiency (21OHD), where precursor steroids, P4 and 17OHP4, are elevated. Our research group previously reported the 5α-reduction and subsequent 3α-reduction of 21dF by the backdoor pathway enzymes, 5α-reductase type 1 and type 2 (SRD5A1 and SRD5A2) and 3α-hydroxysteroid dehydrogenase type 3(3αHSD3/AKR1C2); however, due to the lack of commercially available steroid metabolites, conversions are reported as a decrease in substrate concentrations or as peaks detected in addition to peaks representing substrate in liquid chromatography-mass spectrometry (LC-MS) analysis. In this chapter, we aimed to establish whether 11OHP4 and 11-ketoprogesterone (11KP4) are substrates in the backdoor pathway yielding C11-oxy C_{19} steroids.

11OHA4 has been reported as one of the major C_{19} products of the healthy adrenal gland with increased levels detected post-adrenocorticotropic-releasing hormone (ACTH) stimulation (Rege *et al.* 2013). The C11-oxy C_{19} steroids and 11KP4 were the dominant steroid detected in neonates (Du Toit *et al.* 2018) while C11-oxy androgens have since been reported as the dominant C_{19} steroids in numerous clinical conditions including 21OHD (Turcu *et al.* 2016), 21OHD patients with testicular adrenal rest tumour (TART) (Turcu *et al.* 2017), prostate cancer (PCa) (Du Toit *et*

al. 2017) and polycystic ovary syndrome (PCOS) (O'Reilly *et al.* 2017). Recently, Rege *et al.* 2018 reported 11-ketotestosterone (11KT) as the dominant androgenic steroid detected in girls (age 4-10) with increased 11OHA4, 11-ketoandrostenedione (11KA4), 11OHT and 11KT levels undergoing premature adrenarche (Rege *et al.* 2018). The study was however limited to these four C11-oxy steroids with the C11-oxy C_{21} steroids and C11-oxy C_{19} derivatives undetermined. C11-oxy C_{19} steroids have been reported to be poorly conjugated and would thus remain active in tissue for longer (Du Toit and Swart 2018). Furthermore, serum C11-oxy C_{19} steroid levels, unlike C_{19} steroids, remain constant in women after menopause (Nanba *et al.* 2019) thus, emphasising the importance of understanding the biological impact of C11-oxy steroids.

Increased backdoor pathway activity has been reported in 21OHD (Kamrath *et al.* 2012), PCOS (Marti *et al.* 2017) and PCa. Although CYP11B is primarily expressed in the adrenal, expression has been shown in testes and ovaries (Imamichi *et al.* 2016) as well as testicular tumours (Savard *et al.* 1960). Thus, the biosynthesis of C11-oxy C_{21} steroids are not necessarily limited to the adrenal but extend to the gonads where the C11-oxy C_{21} would be metabolised to C11-oxy C_{19} steroids due to the expression of CYP17A1 together with backdoor pathway enzymes in the testis and ovarian cells. A complete steroid profile and steroid reference ranges are crucial to our understanding of steroidogenesis as well as to identifying abnormalities in steroid profiles which aid the process of diagnosis in clinical conditions as well as in the field of sport where steroids are often abused. The advances in LC-MS and recent increase in steroid profile reports have, yet again, highlighted the prevalence of C11-oxy steroids including C11-oxy C_{19} and C11-oxy C_{21} steroids.

The aim of this chapter was therefore to investigate the biosynthesis and metabolic fate of 11OHP4 with regards to CYP11B enzymes, 11βHSD2 and the enzymes of the backdoor pathway. We investigated the *in vitro* biosynthesis of 11OHP4 from P4 catalysed by CYP11B1 and CYP11B2 as well as the interconversion of 11OHP4 and its keto derivative, 11KP4 by 11βHSD isozymes. The 5α- and subsequent 3α-reduction of 11OHP4 and 11KP4 by the backdoor pathway enzymes were assayed in transiently transfected HEK-293 cells. CYP17A1 activity towards 11KP4 and its metabolites was assessed as well as the metabolism of 11OHP4 and 11KP4 in the LNCaP cell model which expresses the 5α- and 3α-reductases endogenously.

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The *in vitro* metabolism of 11β-hydroxyprogesterone and 11-ketoprogesterone to 11-ketodihydrotestosterone in the backdoor pathway

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ABSTRACT

Increased circulating 11β-hydroxyprogesterone (11OHP4), biosynthesised in the human adrenal, is associated with 21-hydroxylase deficiency in congenital adrenal hyperplasia. 17α-hydroxyprogesterone levels are also increased, with the steroid's metabolism to dihydrotestosterone in the backdoor pathway contributing to hyperandrogenic clinical conditions. In this study we investigated the *in vitro* biosynthesis and downstream metabolism of 11OHP4. Both cytochrome P450 11β-hydroxylase and aldosterone synthase catalyse the biosynthesis of 11OHP4 from progesterone (P4) which is converted to 11-ketoprogesterone (11KP4) by 11β-hydroxysteroid dehydrogenase type 2, while type 1 readily catalysed the reverse reaction. We showed in HEK-293 cells that these C11-oxy C_{21} steroids were metabolised by steroidogenic enzymes in the backdoor pathway–5α-reductase (SRD5A) and 3α-hydroxysteroid type 3 (AKR1C2) converted 11OHP4 to 5α-pregnan-11β-ol,3,20-dione and 5α-pregnan-3α,11β-diol-20-one, while 11KP4 was converted to 5α-pregnan-3,11,20-trione and 5α-pregnan-3α-ol-11,20-dione (alfaxalone), respectively. Cytochrome P450 17α-hydroxylase/17,20-lyase catalysed the hydroxylase and lyase reaction to produce the C11-oxy C_{19} steroids demonstrated in the conversion of alfaxalone to 11-oxy steroids demonstrated in the conversion of alfaxalone to 11ketoandrosterone. In LNCaP cells, a prostate cancer cell model endogenously expressing the relevant enzymes, 11OHP4 and 11KP4 were metabolised to the potent androgen, 11-ketodihydrotestosterone (11KDHT), thus suggesting the C11-oxy C_{21} steroids contribute to the pool of validating the *in vitro* biosynthesis of C11-oxy C_{19} steroids from C11-oxy C_{21} steroids.

The *in vitro* reduction of 11KP4 at C3 and C5 by AKR1C2 and SRD5A has confirmed the metabolic route of the urinary metabolite, 3α,20α-dihydroxy-5β-pregnan-11-one. Although our assays have demonstrated the conversion of 11OHP4 and 11KP4 by steroidogenic enzymes in the backdoor pathway yielding 11KDHT, thus suggesting the C11-oxy C_{21} steroids contribute to the pool of potent androgens, the *in vivo* confirmation of this metabolic route remains challenging.

1. Introduction

Progesterone (P4) and 17α-hydroxyprogesterone (17OHP4) are metabolised by cytochrome P450 21-hydroxylase (CYP21A2) in adrenal steroidogenesis, leading to the production of deoxycorticosterone (DOC) and deoxycortisol, respectively. These steroid metabolites subsequently serve as substrates for cytochrome P450 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2). For decades, the catalytic activity of the CYP11B isozymes have been considered only in terms of DOC and deoxycortisol conversion within the mineralocorticoid and glucocorticoid pathways. However, CYP11B substrates also include other C_{19} and C_{21} steroids –androstenedione (A4), testosterone (T) as well as P4, 17OHP4 and 16α-hydroxyprogesterone (16OHP4) leading to the biosynthesis of C11-oxy C_{19} and C11-oxy C_{21} steroids [1–4]. CYP11B also catalyses the conversion of 17OHP4 to 21deoxycortisol (4-pregnen-11β,17α-diol-3,20-dione or 21dF) after

Abbreviations: P4, progesterone; DHP4, dihydroprogesterone or 5α-pregnan-3,20-dione; 16OHP4, 16αhydroxyprogesterone; 17OHP4, 17α-hydroxyprogesterone; Pdione, 5α-pregnan-17α-ol-3,20-dione; Pdiol, 5αpregnan-3α,17α-diol-20-one; 11OHP4, 11β-hydroxyprogesterone; 11KP4, 11-ketoprogesterone; 11OH-DHP4, 5α-pregnan-11β-ol-3,20-dione; 11K-DHP4, 5α-pregnan-3,11,20-trione; 3,11diOH-DHP4, 5α-pregnan-3α,11β-diol-20-one; alfaxalone, 5α-pregnan-3α-ol-11,20-dione; 21dF, 4-pregnen-11β,17α-diol-3,20-dione or 21-deoxycortisol; 21dE, 4-pregnen-17α-ol-3,11,20-trione or 21-deoxycortisone; 11K-Pdione, 5α-pregnan-11β,17α-diol-3,20-dione; DOC, deoxycorticosterone; CORT, corticosterone; ALDO, aldosterone; DHEA, dehydroepiandrosterone; 3α-Adiol, 5α-androstane-3α,17β-diol; 5α-dione, 5α-androstanedione; 11OH-5α-dione, 11β-hydroxy-5α-androstanedione; 11K-5α-dione, 11-keto-5α-androstanedione; A4, androstenedione; 11OHA4, 11β-hydroxyandrostenedione; 11KA4, 11-ketoandrostenedione; T, testosterone; 11OHT, 11β-hydroxytestosterone; 11KT, 11-ketotestosterone; DHT, dihydrotestosterone; 11OHDHT, 11β-hydroxydihydrotestosterone; 11KDHT, 11-ketodihydrotestosterone; AST, androsterone; 11OHAST, 11β-hydroxyandrosterone; 11KAST, 11-ketoandrosterone

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which the steroid is metabolised by steroidogenic enzymes in the backdoor pathway [4].

11β-hydroxyprogesterone (11OHP4) and 21dF have long been associated with congenital adrenal hyperplasia (CAH), with the former detected at levels significantly lower than 21dF [5–8]. Earlier studies ascribed basal circulating 11OHP4 and 21dF to the 11β-hydroxylation of adrenal P4 and 17OHP4 in 21-hydroxylase deficiency CAH (21CAH) [7] with adrenocorticotropic hormone (ACTH) administration elevating 11OHP4 and 21dF levels [7,8]. We reported CYP11B1 and CYP11B2 catalysing 11OHP4 biosynthesis in a parallel study investigating the 11βhydroxylation of 16OHP4 [3] with both isozymes also catalysing the biosynthesis of 21dF at comparable rates [4].

Testicular adrenal rest tumours (TART) –adrenocortical tumours from adrenal rest tissue which developed within the testis of 21CAH patients, exhibit CYP11B and 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2) activity but not CYP21A2 activity [9] while also expressing the ACTH receptor [9,10]. The production of adrenal-specific steroids, 21dF, 11OHP4 and 11β-hydroxyandrostenedione (11OHA4), by testicular tumours was demonstrated in 21CAH patients [11] with basal 11OHP4 levels in both 21CAH and TART comparable to ACTH-stimulated 11OHP4 levels in normal subjects. ACTH-stimulated 11OHP4 levels were significantly higher in 21CAH [6,7,10–12]. TART tissue was shown to be ACTH-sensitive with 21dF, 11OHP4 and 11OHA4 production increased in patients treated with dexamethasone (DEX) followed by ACTH stimulation [6]. Turcu et al. [13] more recently reported significantly higher levels of 21dF (together with P4, 17OHP4 and 16OHP4) in CAH patients. 11OHP4 levels ranged between 1–11.7 nM, comparable to the peripheral levels of 10 nM reported by Blumberg-Tick et al. [11,13]. 11-ketoprogesterone (11KP4), the product of the 11OHP4 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) catalysed reaction, was not analysed in the aforementioned studies.

The redirection of 17OHP4 to DHT in the backdoor pathway and its contribution to the androgen pool has demonstrated the impact of this C_{21} steroid in 21CAH [14–16]. In the backdoor pathway, initiated by the peripheral conversion of 17OHP4 by the 5α-reductases (SRD5A), cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) catalyses the conversion of the C_{21} steroid, 5α-pregnan-3α,17α-diol-20-one (Pdiol) (the 3α,5α-reduced 17OHP4 product) to the C_{19} steroid, androsterone (AST) (Fig. 1). AST is metabolised by 17β-hydroxysteroid dehydrogenase (17βHSD) isozymes yielding DHT [15]. Interestingly, 11β-hydroxyandrosterone (11OHAST) was also detected, however, the steroid was considered a C_{19} metabolite in ratio analyses [15,16] and not a potential C11-oxy C_{21} or 11OHA4 metabolite. Our *in vitro* studies have shown that 21dF and 21dE are both metabolised in the backdoor pathway to yield 11-ketodihydrotestosterone (11KDHT). 11KDHT is produced from adrenal C11-oxy C_{19} steroids –11OHA4, 11-ketoandrostenedione (11KA4), 11β-hydroxytestosterone (11OHT) and 11-ketotestosterone (11KT) with SRD5A, 11βHSD2 and 17βHSD driving their conversion (supplementary Fig. 1) [2,17]. At 10 nM 11OHT, 11KT, 11OHDHT and 11KDHT activate the androgen receptor, comparable to dihydrotestosterone (DHT) [18].

The metabolism of 11OHP4 and 11KP4 by enzymes in the backdoor pathway has to date not been investigated. 11OHP4, not detected in some of the aforementioned studies, as well as 11KP4 may have been efficiently metabolised by SRD5A, initiating the peripheral metabolism of these C11-oxy C_{21} steroid intermediates in the backdoor pathway (Fig. 1) to produce active androgens. This study therefore aimed to elucidate the *in vitro* biosynthesis of adrenal 11OHP4 and 11KP4 and their metabolism by downstream steroidogenic enzymes characteristic of the backdoor pathway –SRD5A, 3α-hydroxysteroid dehydrogenase type 3 (AKR1C2), CYP17A1 and the 17βHSD isoforms potentially contributing to androgen excess in 21CAH patients. Steroid metabolites were analysed using ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) or ultra-performance convergence chromatography tandem mass spectrometry (UPC^2-MS/MS). Novel steroids were identified by accurate mass determination using quantitative time-of-flight tandem mass spectrometry (Q-TOF MS/MS).

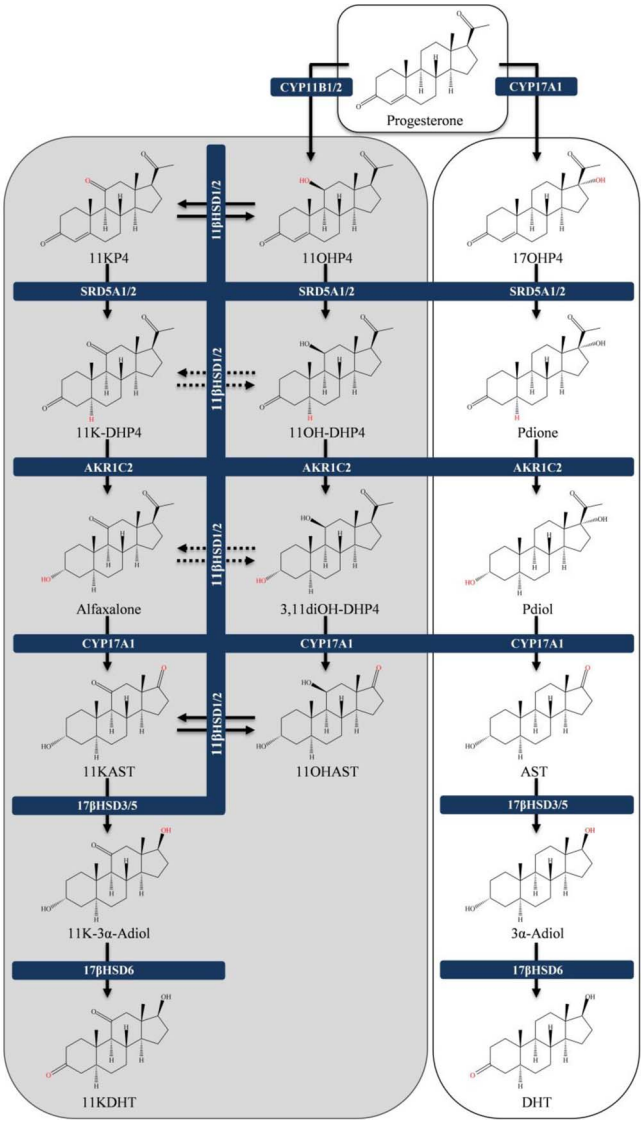

Fig. 1. The biosynthesis of 11-ketodihydrotestosterone from 11β-hydroxyprogesterone and 11-ketoprogesterone by steroidogenic enzymes in the backdoor pathway. Proposed metabolism of 11OHP4 to 11KDHT: shaded; backdoor pathway metabolism of 17α-hydroxyprogesterone to dihydrotestosterone: white.

2. Materials and methods

2.1. Materials

Methyl *tert*-butyl ether (MTBE), Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute 1640 medium (RPMI-1640), β-glucuronidase (Type VII-A from *E. coli*; 5 292 units/mg), D-(+)-Glucose, and steroids (A4, T, 5α-androstanedione (5α-dione), 5α-androstane-3α,17β-diol (3α-Adiol), AST, DHT, P4, 17OHP4, 5α-pregnan-17α-ol-3,20-dione (Pdione), DOC, corticosterone (CORT), aldosterone (ALDO), deoxycortisol, cortisol and cortisone) were purchased from Sigma-Aldrich (St. Louis, USA). Dihydroprogesterone (DHP4), 11OHP4, 11KP4, 5α-pregnan-3,11,20-trione (11K-DHP4), 5α-pregnan-3α-ol-11,20-dione (alfaxalone), Pdiol, 11OHAST, 11-ketoandrosterone (11KAST), 11OHA4, 11OH-5α-dione, 11OHT, 11KA4, 11K-5α-dione, 11KT, 11KDHT, and DHT-glucuronide (DHT-G) were

obtained from Steraloids (Wilton, USA). Deuterated steroid reference standards were purchased from Cambridge isotopes (Andover, USA), these include: deuterated testosterone 1, 2-D2, 98% (D2-T); 9, 11, 12, 12-D4-cortisol; deuterated progesterone 2,2,4,6,6,17A,21,21,21-D9, 98% (D9-P4); deuterated 17α-hydroxyprogesterone 2,2,4,6,6,21,21-D8, 98% (D8-17OHP4); 4-androsten-11β-ol-3,17-dione 2,2,4,6,6,16,16-D7, 98% (D7-11OHA4); and 4-androstene-3,17-dione 2,2,4,6,6,16,16-D7, 97% (D7-A4).

HEK-293 cells were purchased from the American Type Tissue Culture Collection (Manassas, USA). LNCaP cells were purchased from Sigma's European Collection of Cell Cultures (St. Louis, USA). Corning® CellBIND® surface tissue cultureware (T75 flasks, 100 mm dishes, 12- and 24-well plates) were purchased from Corning® Life Science (NY, USA). Trypan blue (0.4%) stain and Countess® cell count plates were purchased from Invitrogen (Eugene, USA) and MColorpHast™ non-bleeding pH-indicator strips from Merck (Darmstadt, Germany). X-tremeGENE HP® DNA transfection reagent was obtained from Roche Diagnostics (Mannheim, Germany). Trypsin-EDTA, fetal bovine serum (FBS) and penicillinstreptomycin were purchased from Oxoid limited (Hampshire, England). Biochrom (Berlin, Germany) supplied the sodium pyruvate and HEPES. A Kinetex PFP column was purchased from Phenomenex Incorporated (Torrence CA). The Promega Wizard® Plus Midipreps DNA purification system (Madison, USA) was purchased from Anatech. All other chemicals and reagents were purchased from reliable scientific supply houses.

2.2. Plasmid constructs

The CYP11B plasmid constructs (human pCMV-CYP11B1 and pCMV-CYP11B2) were obtained from Prof. W.L. Miller (University of California, San Francisco, USA); the human pCR™3–11βHSD1, pCR™3–11βHSD2 and the pcDNA3.2/H6PDH plasmid constructs were purchased from Prof. P. Stewart (University of Leeds, Leeds, England); the human pCMV7-SRD5A1 and pCMV7-SRD5A2 plasmid constructs from Prof. D.W. Russell (University of Texas, Dallas, USA); the human pcDNA3-AKR1C2 plasmid construct from Prof. T.M. Penning (Perelman School of Medicine, University of Pennsylvania, Philadelphia, USA) and the pcDNA3.1-hCYP17A1 plasmid from Prof. A.V. Pandey (University of Bern, Bern, Switzerland). Investigations into the activity of the CYP11B isoforms were performed in the presence of adrenodoxin (ADX) by co-transfecting with the human pTrc99A-ADX plasmid from Prof. R.C. Tuckey (University of Western Australia, Crawley, Australia). The pCIneo plasmid used as a negative control was purchased from Promega (Madison, USA). *E. coli* strain (JM109), transformed with an ampicillin resistant plasmid containing the cDNA encoding appropriate enzymes, was cultured in the presence of ampicillin (100 μg/mL), the vector amplified and purified and used in the transfection of HEK-293 cell. The Promega Wizard® Plus Midipreps DNA purification system (Madison, USA) was subsequently used to isolate and purify the plasmid according to the manufacture's protocol.

2.3. Enzymatic assays in transiently transfected HEK-293 cells

Enzymatic assays were carried out in non-steroidogenic human embryonic kidney (HEK-293) cells transiently transfected with expression vectors containing cDNA encoding CYP11B isoforms, 11β-hydroxysteroid dehydrogenase (11βHSD) and backdoor pathway steroidogenic enzymes –SRD5A, AKR1C2, and CYP17A1. Cells were cultured in DMEM, pH 7.0, supplemented with 0.0015% $NaHCO_3$ ($^{m}/_{v}$), 10% FBS ($^{v}/_{v}$) and 1% penicillin-streptomycin (10,000 U/mL and 10 mg/mL, respectively) at 37 °C, 90% relative humidity and 5% CO_2. After 3–5 passages, confluent cells were replated at 2×10^5 live cells/mL (100 mm dishes and 24-well plates) or at 1×10^5 live cells/mL (12-well plates) and transfected after 24 h. Cells were incubated for a further 48 h after which relevant steroid substrates were added in fresh DMEM and assayed. SRD5A reduced steroids used in this study which were commercially unavailable, 5α-pregnan-11β-ol,3,20-dione (11OH-DHP4) and 11K-DHP4, were prepared as previously described [3]. Media aliquots (500 μL) were collected at specific time intervals and stored at 4 °C until steroid extraction.

2.4. Steroid conversion assays in LNCaP cell model

The potential contribution of 11OHP4 and 11KP4 to the C11-oxy C_{19} androgen pool was subsequently investigated in the androgen-dependent human prostate cancer (LNCaP) cell model. LNCaP cells were cultured in T75 flasks as described in Section 2.3 in RPMI-1640 medium (pH 7) supplemented with 0.0015% $NaHCO_3$ ($^{m}/_{v}$), 0.0025% D (+)-Glucose ($^{m}/_{v}$), 1% penicillin-streptomycin (10,000 U/mL and 10 mg/mL, respectively), 10 mM HEPES, 1 mM sodium pyruvate and 10% FBS ($^{v}/_{v}$). After 3–5 passages, cells were replated into 12-well plates at 2×10^5 live cells/mL, incubated for 48 h after which steroid substrate, 1 μM, was added in fresh RPMI-1640 media and incubated for another 48 h. Due to the presence of endogenous uridine diphosphate glucuronosyltransferase (UGT) enzymes, aliquots (500 μL) were collected in duplicate, of which one set was treated with β-glucuronidase prior to steroid extraction. Sample pH was adjusted to a pH of 6.5 with 1% acetic acid (20 μL/500 μL sample) after which 400 U β-glucuronidase was added. Samples were incubated for 24 h at 37 °C to allow for deglucuronidation of steroids. In order to monitor the deconjugation efficiency, duplicate aliquots of DHT and DHT-G (1 μM) in RPMI-1640, were prepared, one of which was subjected to β-glucuronidase.

2.5. Steroid extraction

Steroids were extracted from media using a liquid-liquid extraction method. Deuterated internal standard mix (1.5 ng D2-T, and 15 ng D4-cortisol, D7-A4, D7-11OHA4, D9-P4 and/or D8-17OHP4) was added to all samples prior to the addition of MTBE (3:1). Samples were vortexed for 15 min, the aqueous phase frozen at 80 °C for ~20 min and the organic phase removed and dried under nitrogen gas (N_2) at 40 °C. Steroid residues were resuspended in HPLC-grade methanol (50%) and analysed using either UHPLC-MS/MS or UPC^2-MS/MS.

2.6. Identification, accurate mass determination and quantification of steroid metabolites

Commercially unavailable steroids were prepared by incubating precursor steroids at high substrate concentrations (10 μM) in HEK-293 cells transiently transfected with either SRD5A or AKR1C2. Steroid metabolites were extracted as described and the accurate molecular mass and product ions were determined using a Q-TOF mass spectrometer (Milford, USA). Steroids were separated on a Waters BEH C18 column (2.1 mm × 100 mm, 1.7 μm) with a mobile phase consisting of 1% formic acid in acetonitrile. The molecular and product ion values were subsequently used to analyse and quantify the steroids using either UHPLC-MS/MS or UPC^2-MS/MS. Steroid metabolites were quantified using reference steroid standards to generate standard curves which were run in series with the samples being analysed. Metabolites representing steroids for which reference steroids standards were unavailable commercially could not be quantified and, when detected were depicted as the steroid response (or area under the peak) which was generated by die mass spectrometer from the ion count for the specific steroid.

Steroid conversion assays were analysed using UHPLC-MS/MS with steroids being separated on an ACQUITY UHPLC (Waters, Milford, USA) using a Phenomenex UHPLC Kinetex PFP column (2.1 mm × 100 mm, 2.6 μm) (Torrance, CA, USA), coupled to a Xevo triple quadrupole mass spectrometer (Waters, Milford, USA). Steroid stocks (2 mg/mL), dissolved in absolute ethanol, were used in preparation of a standard range (0.002–2.5 ng/μL) in appropriate experimental culture media from which 500 μL aliquots were extracted as previously described in Section

2.5.

Analyses of steroid metabolism in LNCaP cells was carried out using the ACQUITY UPC^2-MS/MS (Waters, Milford, USA) and an ACQUITY UPC^2 BEH column (3 mm × 100 mm, 1.7 µm) operating in multiple reaction monitoring (MRM) and positive electrospray ionisation (ESI+) modes. All steroid quantifications were referenced to a standard range (0.0001–1 ng/µL) prepared in experimental culture media and extracted as previously described. Chromatography conditions and mass spectrometry parameters are summarised in the supplementary data file.

2.7. Statistical analysis

Masslynx Software 4.1 assisted in all MS data collection and steroid analyses. Statistical relevance of the results was analysed using GraphPad Prism 6 (GraphPad Software, Inc., CA, USA) by two-way ANOVA followed by Tukey's multiple comparisons test and expressed as means ± SD. Statistical significance was measured as a probability (p) value ≤ .05 ($^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$, $^{****}p \leq .0001$).

3. Results

3.1. Identification and characterisation of 11OHP4 and 11KP4 metabolites

Prior to investigations into the C11-oxy C_{21} steroids, the downstream SRD5A and AKR1C2 metabolites for which reference standards are not commercially available, were prepared and analysed. Accurate mass determinations of the novel metabolites were carried out using Q-TOF-MS. The SRD5A catalysed steroid conversion of 11OHP4 and 11KP4, and the subsequent AKR1C2 catalysed conversion of the 5α-reduced products were carried out in transiently transfected HEK-293 cells. Conversion assays were conducted using 10 µM to ensure sufficient formation of product –5α-reduced metabolites, 11OH-DHP4 and 11K-DHP4 and the 3α,5α-reduced metabolites, 5α-pregnan-3α,11β-diol-20-one (3,11diOH-DHP4) and alfaxalone. The accurate molecular mass and product mass transitions of the C11-oxy C_{21} steroid metabolites were confirmed in ESI+ mode and were subsequently used in UHPLC-MS/MS and UPC^2-MS/MS analyses of metabolites produced in steroid conversion assays. The theoretical and observed molecular mass $[M + H]^+$ and product ions are summarised in Table 1 and the spectra and chemical structures presented in Fig. 2.

3.2. The 11β-hydroxylation of P4 by CYP11B1 and CYP11B2

The *in vitro* biosynthesis of 11OHP4 by CYP11B1 and CYP11B2 was investigated in non-steroidogenic HEK-293 cells. Included in the study were A4 and T as well as the natural substrates, deoxycortisol and DOC.

A notable difference in the catalytic activity of the two enzymes towards P4 was observed in which CYP11B1 catalysed the hydroxylation of 1 µM P4 more readily than CYP11B2 with the formation of 0.66 µM 11OHP4 by CYP11B1 while the CYP11B2-catalysed production reached 0.37 µM at 12 h (Fig. 3A).

CYP11B1 also catalysed the hydroxylation of both A4 and T to their respective C11-hydroxyl products (Fig. 3B and C) more efficiently than CYP11B2, with 0.715 µM 11OHA4 and 0.58 µM 11OHT being detected after 12 h. In contrast, the production of 11OHA4 by CYP11B2 was only 0.37 µM, corroborating findings previously reported by Swart et al. (2013), while 11OHT production reached 0.22 µM.

Assays with the natural substrates showed CYP11B1 catalysing the conversion of both deoxycortisol and DOC efficiently yielding 0.91 µM cortisol and 0.63 µM CORT, respectively, at 12 h. The conversion of deoxycortisol by CYP11B2 was less efficient yielding 0.46 µM cortisol while 80% DOC was converted by CYP11B2 yielding 0.31 µM CORT and 0.035 µM ALDO. The intermediary product of the conversion of CORT to ALDO, 18hydroxycorticosterone, was not analysed.

3.3. The interconversion of 11OHP4 and 11KP4 by 11βHSD type 1 and type 2

CYP11B metabolites, such as cortisol and 11OHA4, serve as substrates for 11βHSD2 which catalyses their conversion to the C11-keto derivatives, cortisone and 11KA4, respectively, with 11βhydroxysteroid dehydrogenase type 1 (11βHSD1) catalysing the opposite reaction [2,19]. Even though 11OHP4 is generally considered to be an inhibitor of 11βHSD2, we assayed the catalytic activity of the 11βHSD isoforms towards 11OHP4 and 11KP4 in HEK-293 cells co-transfected with either 11βHSD1 and hexose-6-phosphate dehydrogenase (H6PDH) or 11βHSD2 and pCI-neo.

The conversion of 11OHP4 by 11βHSD2 (Fig. 4A) was markedly lower than the conversion of 11KP4 by 11βHSD1 (Fig. 4B), 50% at 22 h and 8 h, respectively, with only 0.2 µM substrate remaining after 20 h in the latter assay.

3.4. The 5α-reduction of 11OHP4 and 11KP4 by SRD5A1 and SRD5A2

The 5α-reduction of 11OHP4 and 11KP4 to 11OH-DHP4 and 11K-DHP4 was subsequently investigated in HEK-293 cells. 11OHP4 and 11KP4 were initially assayed at 10 µM for product identification (Section 3.1) and had shown full conversion by both SRD5A1 and SRD5A2 after 12 h (supplemental Fig. 2) with no remaining substrate being detected.

The conversion of 5 µM 11OHP4 by SRD5A (Fig. 5A) showed that the substrate was fully converted by both isozymes at 3 h, with 50% conversion reached after 15 and 45 min by SRD5A1 and SRD5A2, respectively. The initial reaction rate of SRD5A1 was 13.24 µM/hr and that of SRD5A2 was 5.063 µM/hr. The metabolism of 11KP4 (Fig. 5B) catalysed by both isozymes resulted in full conversion at 3 h with 50% conversion being reached after 30 and 50 min by SRD5A1 and SRD5A2, respectively. The initial reaction rate of SRD5A1 was 7.736 µM/hr and that of SRD5A2 was 4.355 µM/hr. Although no remaining substrate was detected, the 11K-DHP4 yield was 3.98 µM (SRD5A1) and 4.16 µM (SRD5A2). These data indicate possible endogenous conversion of the formed product. The conversion of T to DHT by SRD5A (supplemental Fig. 3) which was assayed in parallel as a positive control showed T's full conversion, with DHT formation correlating to decreased T concentrations. Collectively, the data indicate the rapid metabolism of 11OHP4 and 11KP4 by both SRD5A isozymes.

3.5. The reduction of 11OH-DHP4 and 11K-DHP4 by AKR1C2

Once 11OHP4 and 11KP4 are metabolised by SRD5A in the backdoor pathway, their 5α-reduced metabolites would be converted by

Table 1
Molecular ion and product ion species of the SRD5A and AKR1C2 products of 11OHP4 and 11KP4.

Steroid	Theoretical mass $[M + H]^+$	Observed accurate mass $[M + H]^+$	Chemical formula	Product ion $[M + H]^+$
5α-pregnan-11β-ol-3,20-dione (11OH-DHP4)	333.2430	333.2433	$C_{21}H_{32}O_3$	173; 84.8
5α-pregnan-3,11,20-trione (11K-DHP4)	331.2273	331.2278	$C_{21}H_{30}O_3$	147; 105
5α-pregnan-3α,11β-diol-20-one (3,11diOH-DHP4)	335.2686	335.2589	$C_{21}H_{34}O_3$	241.2; 159; 119.2
5αpregnan-3α-ol-11,20-dione (alfaxalone)	333.2430	333.2421	$C_{21}H_{32}O_4$	159; 147; 85

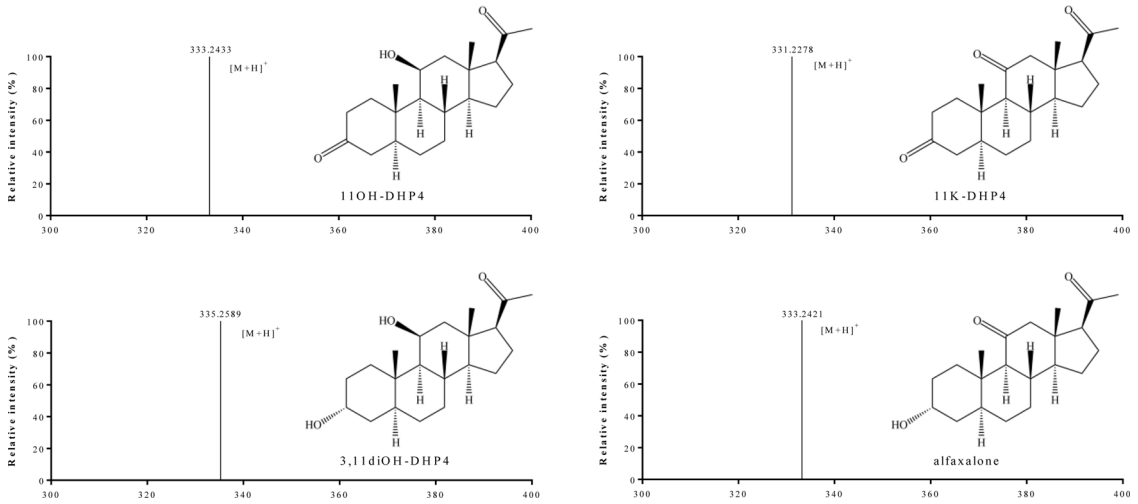

Fig. 2. Mass spectrometry spectra of reduced C11-oxy C_{21} steroids $[M + H]^+$ and their chemical structures. Spectra depict the relative abundance (%) of a single injection of the steroid sample.

AKR1C2 which catalyses the reduction of the C3 keto-group. The catalytic activity of AKR1C2 towards 11OH-DHP4 and 11K-DHP4 to yield 3,11diOH-DHP4 and alfaxalone, respectively, was therefore assayed.

11OH-DHP4 was prepared as previously described and the conversion of 11OHP4 by SRD5A was analysed prior to assaying the catalytic activity of AKR1C2 towards 11OH-DHP4. LC–MS analyses confirmed that 11OHP4 had been fully converted after 12 h with no substrate being detected (data not shown). The production of 3,11diOH-DHP4 in the subsequent conversion of 11OH-DHP4 by AKR1C2 was analysed after 24 h (Fig. 6). A significant loss in the 11OH-DHP4 signal response

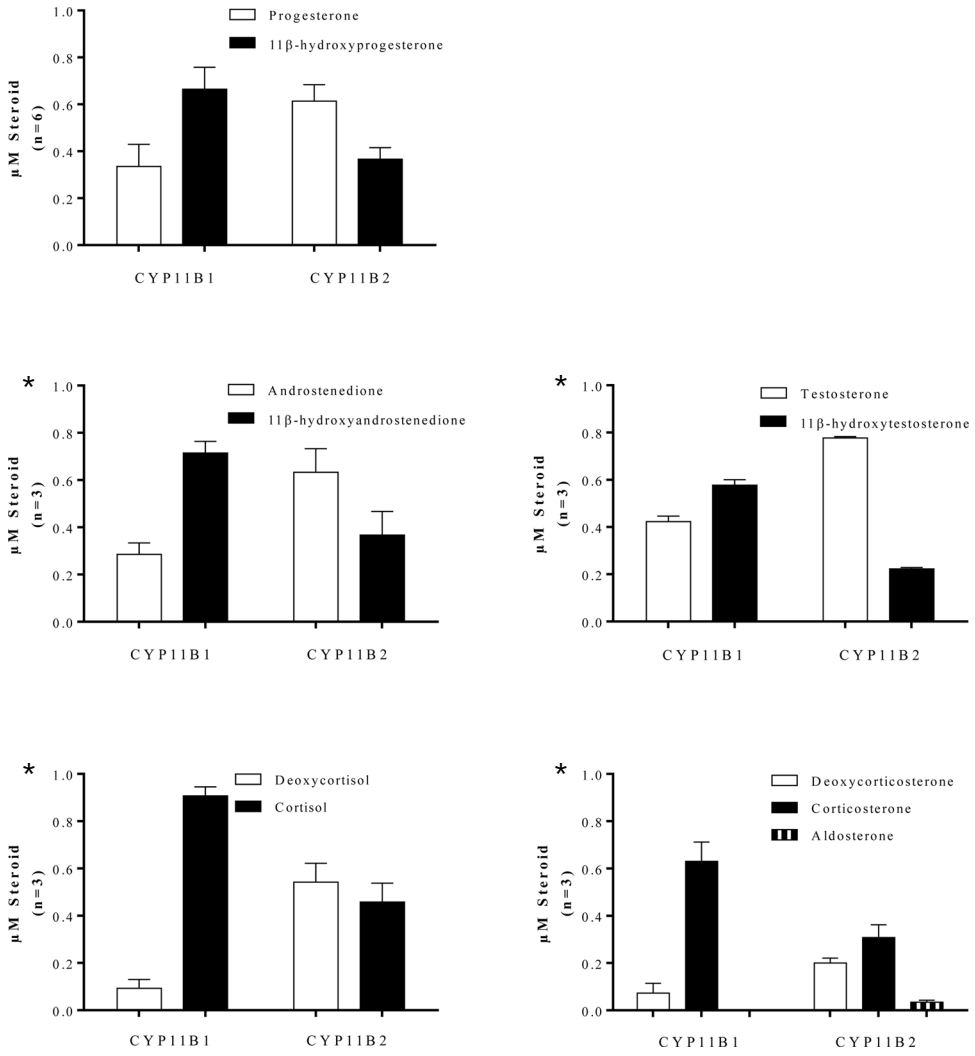

Fig. 3. C11-oxy C_{21} and C11-oxy C_{19} are biosynthesised by CYP11B1 and CYP11B2. Substrate, white bar; product, black bar. Results are representative of two and/or three independent experiments shown as the mean ± SD.

* Reproduced from: Van Rooyen, D., 2017. 'The metabolism of adrenal 11b -hydroxyprogesterone, 11keto-progesterone and 16Ŭ-hydroxyprogesterone by steroidogenic enzymes', MSc thesis, University of Stellenbosch, South Africa.

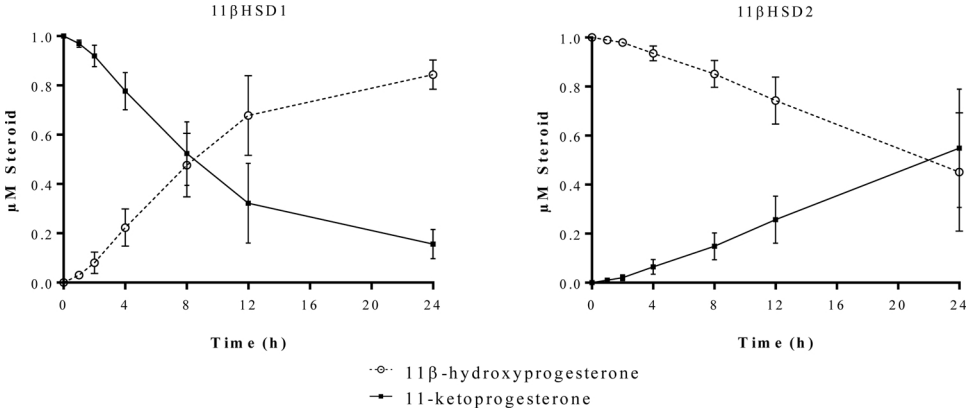

Fig. 4. The interconversion of 11β-hydroxyprogesterone and 11-ketoprogesterone is catalysed by the 11βHSD isoforms. Results are expressed as the mean ± SD of two independent experiments performed in triplicate (n = 6).

(steroid area/internal standard area) was detected and, although not quantifiable, a gain in the 3,11diOH-DHP4 signal response was detected when compared to the initial concentration (T_0) and negative pCI-neo control sample signals, confirming the production of 3,11diOH-DHP4.

Investigations into the metabolism of 11K-DHP4 to alfaxalone by AKR1C2 was facilitated due to the steroids being commercially available allowing quantification. 11K-DHP4 levels were significantly decreased after 24 h yielding 0.415 µM alfaxalone (Fig. 7). Low levels of alfaxalone was detected in the pCI-neo sample, but was shown to be statistically insignificant. It is possible that the alfaxalone formed in the control reaction may be due to the endogenous aldo-keto reductase activity [20]. The conversion of DHT to 3α-Adiol was assayed in parallel as a positive control and showed full conversion after 24 h (supplemental Fig. 4).

3.6. The metabolism of 11KP4, 11K-DHP4 and alfaxalone by CYP17A1

In the backdoor pathway, the 17,20-lyase activity of CYP17A1 converts the C_{21} steroids to C_{19} steroids in which the 3α,5α-reduced product of 17OHP4 (Pdiol) is lysed by CYP17A1 yielding AST. The latter is subsequently metabolised by the 17βHSD enzymes to DHT. In the conversion of the reduced 11OHP4 and 11KP4 metabolites to their respective C_{19} steroids in the backdoor pathway, both the17α-hydroxylase and the 17,20-lyase activity of CYP17A1 would be required as both intermediates lack the hydroxyl group at C17. Determining the conversion of 3,11diOH-DHP4 by CYP17A1 is hampered by the commercial unavailability of the steroid together with AKR1C2 not fully converting the 5α-reduced precursor steroid. Our investigation into the hydroxylase and lyase activity of CYP17A1 towards the 3α,5α-reduced intermediates was therefore carried out only with alfaxalone, the 3α,5α-reduced product of 11KP4. We assayed the conversion of 11KP4, 11K-DHP4 and alfaxalone by human CYP17A1 and included Pdiol as a positive control to monitor the conversion to AST.

After 12 h the conversion of 11KP4 yielded ~0.55 µM 21-deoxycortisone (4-pregnen-17α-ol-3,11,20-trione or 21dE) with ~0.43 µM substrate remaining and negligible levels of the lyase product, 11KA4 (1%) (Fig. 8A). The conversion of 1 µM 11K-DHP4 (Fig. 8B) by

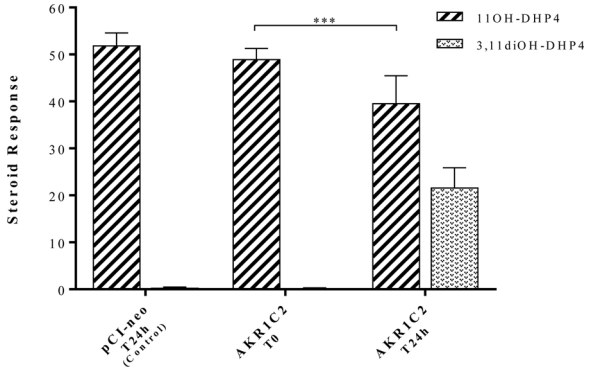

Fig. 6. AKR1C2 catalyses the 3α-reduction of 5α-pregnan-11β-ol-3,20-dione to 5α-pregnan-3α,11β-diol-20-one. Results represent MS steroid response signal and expressed as the mean ± SD of two independent experiments performed in triplicate (n = 6). Statistical analysis was carried out using a two-way ANOVA followed by a Tukey's multiple comparison test.

CYP17A1 showed a significant decrease in its concentration to ~0.69 µM, with the formation of the 17α-hydroxylated intermediate, 5α-pregnan-3α,17-diol-11,20-dione (11K-Pdione) being detected. The subsequent formation of 11K-5α-dione, the lyase product of the intermediate, was negligible. In the conversion of alfaxalone (Fig. 8C) ~0.42 µM substrate remained after 12 h, yielding ~0.58 µM 11KAST, indicating that CYP17A1 catalysed both the hydroxylation at C17 followed by the 17,20 lyase reaction. In the control assays, Pdiol was readily converted to AST in the 17,20 lyase CYP17A1 catalysed reaction, with only ~5% Pdiol remaining (Fig. 8D). Taken together, 11KP4, 11K-DHP4 and alfaxalone were all metabolised by CYP17A1.

3.7. 11OHP4 and 11KP4 metabolism in the LNCaP cell model

In the data presented above it was shown that the C11-oxy C_{21} steroids are metabolised by steroidogenic enzymes –11βHSD, SRD5A, AKR1C2, and CYP17A1 expressed individually in a heterologous

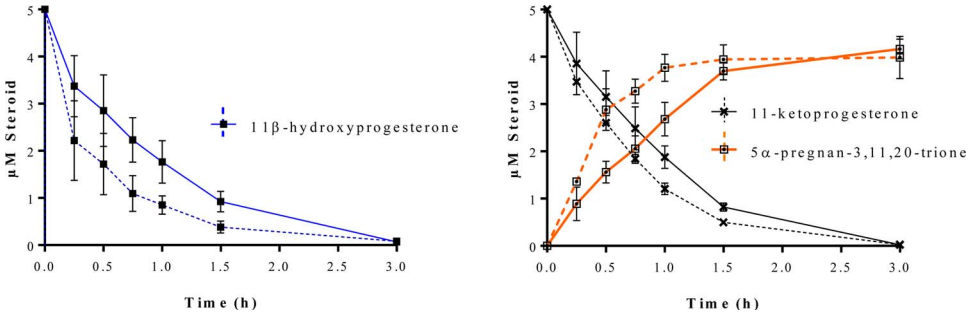

Fig. 5. SRD5A catalyses the 5α-reduction of 11β-hydroxyprogesterone and 11-ketoprogesterone. SRD5A1, dashed line; SRD5A2, solid line. Results are expressed as the mean ± SD of two independent experiments each performed in triplicate (n = 6).

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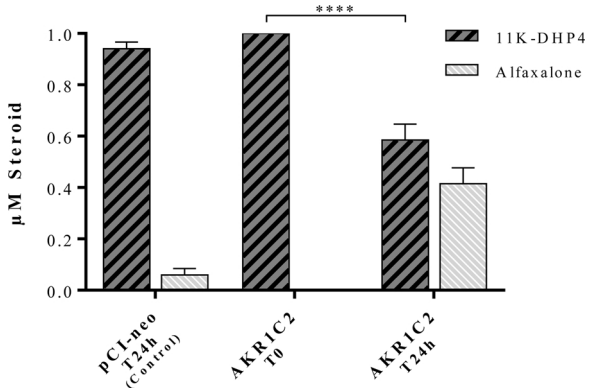

Fig. 7. AKR1C2 catalyses the 3α-reduction of 5α-pregnan-3,11,20-trione to alfaxalone. Results are expressed as the mean ± SD of two independent experiments performed in triplicate (n = 6). Statistical analysis was done using a two-way ANOVA followed by a Tukey's multiple comparison test.

system. In order to assess their metabolism in the context of competing enzymes, the metabolism of the C11-oxy C_{21} steroids was investigated in a cell model system expressing the enzymes of interest. The metabolism of 1 µM 11OHP4 and 11KP4 was therefore assayed in LNCaP cells, a castration-resistant prostate cancer cell model which expresses all the relevant steroidogenic enzymes [21]. Steroids were deconjugated after which quantifiable steroid metabolites were analysed using UPC^{2}MS/MS. Analyses of unconjugated and total (unconjugated and conjugated) steroid levels showed that there was no significant difference for any of the quantified steroid metabolites (not shown), indicating that the C11-oxy steroids were not glucuronidated. Analysis of 11OHP4 metabolites (Fig. 9A) showed more than 30% 11OHP4 was metabolised to yield 11KP4 (42 nM) (catalysed by 11βHSD2), which was subsequently metabolised to 11K-DHP4 (4.5 nM) (catalysed by SRD5A) and to alfaxalone (63.6 nM) (catalysed by AKR1C2) and 11KDHT (6.9 nM) (catalysed by CYP17A1 and 17HSD) with 0.690 µM 11OHP4 detected after 48 h. The metabolism of 11KP4 (Fig. 9B), yielded 11K-DHP4 (62.0 nM), alfaxalone (72.0 nM) and 11KDHT (3.5 nM) with 0.609 µM 11KP4 in its unconjugated, free form.

4. Discussion

11OHP4 has received less attention than 21dF in studies focussing on 21CAH and is perhaps deemed of lesser importance due to the steroid being present in circulation at far lower concentrations than 21dF (pmol/L vs nmol/L) and, at times, undetectable. The biosynthesis of both is catalysed by CYP11B1 and CYP11B2 however, the production of 11OHP4 *in vivo* would thus seem more likely to occur in the zona glomerulosa where CYP17A1 is absent, in contrast to its biosynthesis in the zona fasciculata where CYP11B1 would compete with CYP17A1 for P4. Circulating 11OHP4 levels are low –adults (18 pmol/L) and prepubertal children (21 pM), however, increasing ~4.5-fold upon ACTH stimulation. Lower 11OHP4 levels were detected in the non-salt-wasting (nSW) than in the salt-wasting (SW) phenotype with high plasma renin activity in patients undergoing treatment for 21CAH, suggesting 11OHP4 production depended on the degree of 21-hydroxylase deficiency together with AngII stimulation [7,8]. Analyses of urinary metabolites in 21CAH neonates has indeed shown that there is an induction in the mineralocorticoid pathway with a greater increase in the 3α,5β,20α metabolite of 11KP4 compared to that of the 21dF metabolite [22].

Few studies have reported on circulating 11OHP4 with the analyses of 11KP4 or their metabolites rarely included. Considering the C11-oxy C_{19} steroids –with both the hydroxyl and keto forms identified in adults, circulating levels were significantly higher in 21CAH compared to control subjects. In both groups 11KT was ~3-fold higher than 11OHT while 11OHA4 levels were higher than 11KA4 levels [23] –indicative of not only 11OHT's conversion to 11KT by 11βHSD2, but also of 11OHA4's conversion to 11KA4 and to 11KT (catalysed by 17βHSD). While the interconversion of 21dF and 21dE by the 11βHSD isoforms is comparable [4], the conversion rate of 11KP4 by 11βHSD1 was 2.5-fold faster than 11βHSD2's conversion of 11OHP4. Together with the very efficient 5α-reduction of 11OHP4 and of 11KP4 by both SRD5A isoforms, our *in vitro* data suggest that *in vivo* 11OHP4 may serve primarily as a peripheral substrate for SRD5A. The higher initial reaction rate

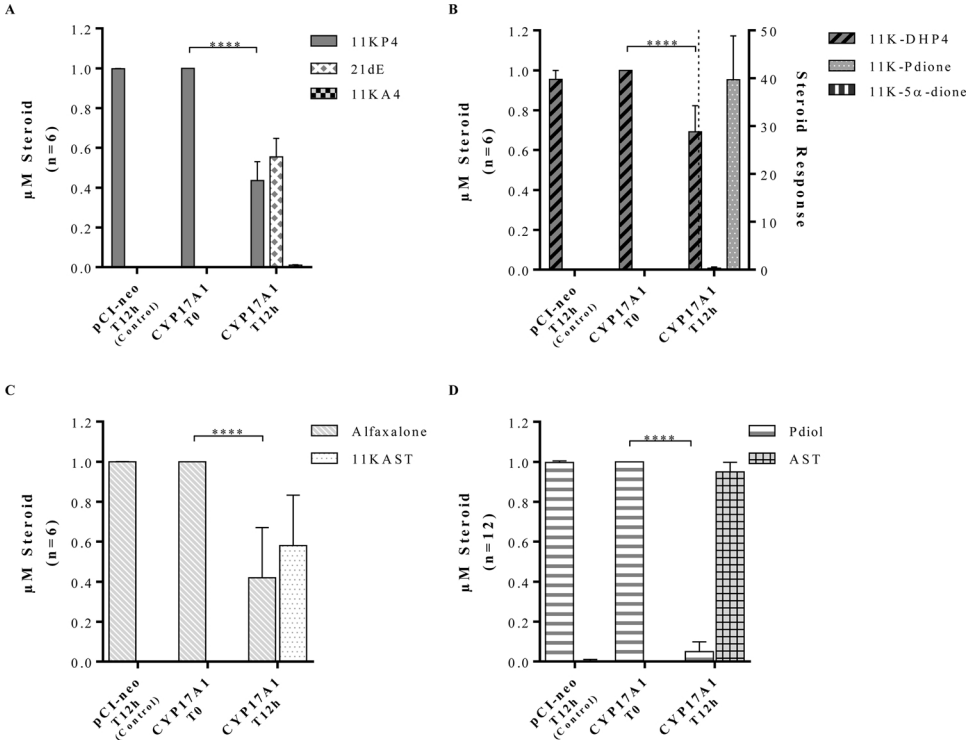

Fig. 8. CYP17A1 catalyses the conversion of C11-oxy C_{21} steroids. Conversion of (A) 11-ketoprogesterone to 21-deoxycortisone and 11-ketoandrostenedione; (B) 5α-pregnan-3,11,20-trione to 5α-pregnan-11β,17α-diol-3,20-dione and 11-keto-5α-androstanedione; (C) alfaxalone to 11-ketoandrosterone and (D) 5α-pregnan-3α,17α-diol-20-one to androsterone. Results are expressed as the mean ± SD. Statistical analysis was carried out using a two-way ANOVA followed by a Tukey's multiple comparison test.

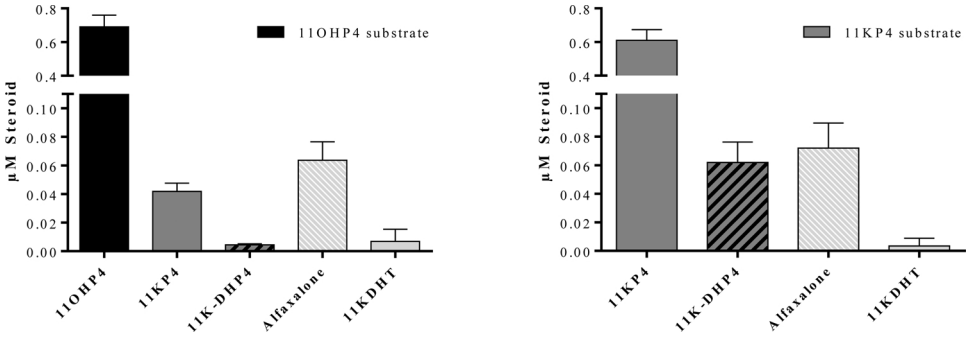

Fig. 9. 11β-hydroxyprogesterone and 11-ketoprogesterone are metabolised to 11-ketodihydrotestosterone in LNCaP cells. Results depict total steroids (free + conjugated), expressed as the mean ± SD of an experiment performed in triplicate (n = 3).

exhibited by SRD5A1 for 11OHP4 (2.6-fold) and 11KP4 (1.8-fold) compared to SRD5A2, underscores the biological relevance of these C11-oxy C_{21} in diseases associated with elevated SRD5A1 expression *viz.* prostate cancer [24–27], endometrial cancer [28], breast cancer [29], ovarian cancer and polycystic ovarian syndrome (PCOS) [30,31]. SRD5A1 is upregulated in PCOS ovarian tissue together with the steroidogenic enzymes in the backdoor pathway, including CYP17A1. Authors concluded that this pathway may contribute to hyperandrogenism in women [32], a scenario in which 11OHP4 would compound the contribution of the backdoor pathway to androgen levels, adding 11KDHT to the androgen pool.

The efficient reduction of 11OHP4 and 11KP4 by SRD5A suggests that these steroids may contribute to elevated androgen levels in 21CAH, as in the case of 17OHP4 which is reduced by SRD5A and channelled to DHT via the backdoor pathway. Our *in vitro* studies showed the 5α-reduction of 21dF and 21dE and conversion in the backdoor pathway yielded C11-oxy C_{19} steroids [4]. Both the 5α-reduced products, 11OH-DHP4 and 11K-DHP4, were converted by AKR1C2 yielding 3,11diOH-DHP4 and alfaxalone, respectively (Fig. 1). We quantified only 3α-reduced 11KP4 metabolites due to the availability of reference standards and showed significant conversion of 11K-DHP4 to alfaxalone (40%). While steroid receptor interactions of the C11-oxy C_{21} steroids have yet to be determined, their 3α,5α-reduced metabolites, once converted by CYP17A1 to C_{19} intermediates and subsequently by the 17βHSDs, would yield 11KDHT (Fig. 1) capable of activating the androgen receptor. However, the conversion by CYP17A1 is key in converting 11OHP4 and 11KP4 intermediates to the C11-oxy androgens –both the 17α-hydroxylase and 17,20-lyase activities. The conversion of 17OHP4 to DHT requires the lyase activity only although Gupta et al. [14] showed rapid conversion of allopregnanolone (3α,5α-reduced form of P4) to AST demonstrating both hydroxylase and lyase activities of CYP17A1 [14]. These data showed that the C3-hydroxyl group and reduced C4-C5 double bond enable the more promiscuous catalytic activity of CYP17A1 towards substrates other than P4 and pregnenolone. Our data which showed the 17αhydroxylation of 11K-DHP4 yielding 11K-Pdione with no lyase product detected, demonstrates only hydroxylase activity (Fig. 8A and B) with the C3keto moiety in place. The requirement of both the C3-hydroxyl group and reduced C4-C5 double bond for the lyase reaction was confirmed with alfaxalone which readily yielded 11KAST (Fig. 8C). Similar findings in a study by Gupta et al. [14] had led authors to suggest that the C3-hydroxyl moiety is a prerequisite for the 17,20-lyase activity, which our studies have corroborated. Of interest is that 11KP4 appears to be a better substrate than 11OHP4, with ~60% being converted to 21dE and negligible lyase product, 11KA4, being formed.

The contribution of 11OHP4 and 11KP4 to the androgen pool *in vivo* may be relevant in disorders characterised by increased C11-oxy C_{21} steroids as well as in normal human sexual development, adrenarche and perhaps even in mini-puberty. Reports are limited with recent studies suggesting that the *in vivo* flux of 11OHP4 is towards 11KP4 production. In healthy infants urinary 11KP4 is markedly higher than 11OHP4 in the first year of life –exhibiting sex specificity with higher 11KP4 levels in girls and higher 11OHP4 levels in boys. 11KP4 and 11OHAST furthermore increased over the oneyear period [33]. In support, an earlier study had identified the downstream urinary metabolite of 11KP4, 5β-pregnan-3α,20α-diol-11-one, attributing its origin to the 11β-hydroxylation of P4 in the zona glomerulosa of 21CAH neonates –a study in which they also showed the shift from the 5β-reduction to the 5α-reduction after the first 4 weeks of life [34]. Given the increase in 5α-reduction leading up to mini-puberty –the period spanning month one to three in male neonates, and androgen biosynthesis reportedly favouring the backdoor pathway over the classic pathway during mini-puberty [35], the C11-oxy C_{21} steroids could potentially impact the developmental process.

The contribution of both the classic and backdoor pathways in normal male sexual differentiation has been demonstrated in mutations of the genes encoding AKR1C2 and AKR1C4 which result in disordered sexual development [36,37]. In LNCaP cells which expresses the relevant enzymes [22], 21dF metabolism yielded 11OHAST and 11KAST [4] and while 11OHAST was not detected in the present study 11KDHT was, indicating biosynthesis via 11KAST (Fig. 1). Our cumulative *in vitro* data suggest that the 11OHAST metabolite detected in infants reported by Dhayat et al. [33] may either be a metabolite of the C11-oxy C_{21} steroids and/or 11OHA4. In the metabolism of both 11OHP4 and 11KP4 in LNCaP cells, alfaxalone was the major metabolite indicative of endogenous 11βHSD2, SRD5A and AKR1C2 expression. Both assays produced 11KDHT suggesting conversion of either 3,11diOH-DHP4 or alfaxalone by CYP17A1 and subsequently by the 17βHSD isozymes, providing evidence of the *in vitro* conversion of 11OHP4 and 11KP4 to C_{19} steroids in the backdoor pathway.

In vivo evidence of C11-oxy C_{21} an d C11oxy C_{19} metabolites in the backdoor pathway, lie in 21dE, 11KP4, 11OHA4 and 11KA4 having been identified in neonatal urinary samples using GC–MS/MS [34,38,39]. While neither 11OHA4 nor 11KA4 were detected, their 3α,5α- and 3α,5β-metabolites were, together with other downstream metabolites. The 3α,5α metabolites were significantly increased ($P < .001$) [39] while the 3α,5β derivative of 21dE was a major metabolite in 21CAH neonates [38]. In addition, the 3α,5β,20α metabolite of 11KP4 was 100-fold higher in 21CAH neonates in the period spanning 8–27 days compared to levels in the first week of life. Data analyses showed that while the 5α-reduction of both 11OHA4 and 11KA4 was predominant over the 5β-reduction, the reduced metabolites of 21dE were predominantly present in the 5β form with the 5α-reduction only increasing later [34]. It is thus likely that after the first month 11OHP4, 11KP4, 21dF and 21dE would yield active C11-oxy C_{19} steroids *in vivo* via the backdoor pathway in newborns while 11OHA4 may well be adding to the pool of active androgens onwards from birth. Kamrath et al. [40] recently reported 11OHAST to be the main urinary steroid metabolite in children (under 18 years) receiving hydrocortisone treatment for 21CAH. Based on AST and etiocholanolone excretion levels being similar, they concluded that the backdoor pathway appeared not to play a role in treated children [40]. It is probable that 11OHP4, 11KP4, 21dF and 2dE would have contributed, in addition to 11OHA4, to the elevated 11OHAST levels and would thus

undoubtedly implicate the backdoor pathway. Although we have shown with this *in vitro* study that 11OHP4 and 11KP4 can be metabolised in the backdoor pathway, only the 3α,5α- and 3α,5β-metabolites of 11KP4 are *in vivo* evidence of these adrenal C11-oxy C_{21} steroids and whether these metabolites will be converted to 11OHAST, or 11KAST and subsequently to 11KDHT and contribute to circulating androgen levels remains to be determined *in vivo*.

5. Conclusion

Whether the backdoor pathway prevails in normal sexual differentiation and physiological development or in hyperandrogenicity in CAH and/or 21CAH can only be fully evaluated once all contributing steroid metabolites have been assessed *in vivo* –including the C11-oxy C_{19} and the C11-oxy C_{21} steroids and their full spectrum of metabolites. There can be little doubt as to the contribution of these C11-oxy steroids to normal development and to disease as is evidenced in the urinary presence of the C11-oxy C_{21} steroids and their metabolites as well as the metabolic pathways involved. The presence of these C11-oxy steroids does indeed pose a plethora of unanswered questions. Metabolic routes need to be fully explored –specifically the potential contribution of the C11-oxy C_{21} steroids to the C11-oxy C_{19} steroid pool via the backdoor pathway that our *in vitro* study has shown. It may be possible that once *in vivo* pathways have been fully elucidated and characterised, distinctive metabolites associated with clinical conditions may be identified leading to more accurate cost-effective diagnoses without the need for complex steroid metabolomics. Making sense of an overabundance of analytical data employing complex ratios may even become a thing of the past.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2017.12.014

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Supplementary data

Supplementary table 1

UHPLC-MS/MS gradient applied in the separation of steroids listed in supplementary table 2. Injection volume was set to 5 µL, and the flow rate 0.4 mL/min.

	Time (min)	**%A***	**%B****	**Curve**
1	initial	85.0	15.0	Initial
2	1.00	60.0	40.0	6
3	3.50	45.0	55.0	6
4	3.60	0.0	100.0	6
5	4.00	0.0	100.0	6
6	4.01	85.0	15.0	6
7	5.00	85.0	15.0	6

Supplementary table 2

UHPLC-MS/MS separation and quantification of C_{21} steroids. The gradient described in supplementary table 1 was used to separate the steroids tabulated below. Each steroid is presented with its retention time (RT), molecular (parent) and product (daughter) ion species together with the respective mass spectrometry properties: cone voltage (CV), collision energy (CE), calibration range (ng/µL) and the linearity (r^2). The limit of quantification (LOQ) is 0.02 ng/µL.

Steroid	RT (min)	Mass transitions			Cone Voltage (V)			Collision Energy (eV)			Calibration range (ng/µL)	Linearity (r^2)
		Product ion 1	Product ion 2	Product ion 3								
Internal standards												
D2-T	3.17	291.00 > 99.10	291.00 > 111.25	-	25	25		30	30		-	-
D8-17OHP4	3.22	340.10 > 100.00	340.10 > 114.00	-	26	26		25	28		-	-
D9-P4	4.23	324.20 > 100.00	324.20 > 113.00	-	30	30		20	25		-	-
C_{21} steroids												
11KP4	2.98	329.21 > 121.00	329.21 > 84.80	-	15	15		20	20		0.02 - 2	0.9916
11K-DHP4	3.31	331.20 > 105.00	331.20 > 147.00	-	25	25		30	30		0.02 - 2	0.9939
Unknown	2.81	333.20 > 84.80	333.20 > 297.10	-	15	15		20	15		0.02 - 2	-
Alfaxalone	3.48	333.20 > 147.20	333.20 > 159.27	-	25	25		30	30		0.02 - 2	0.9903
11OHP4	3.03	331.20 > 121.00	331.20 > 295.00	-	20	20		25	15		0.02 - 2	0.9916
11OH-DHP4	3.34	333.10 > 84.80	333.10 > 173.00	-	30	30		30	20		0.02 - 2	-
16OHP4	2.60	331.20 > 97.00	331.20 > 108.90	-	30	30		15	15		0.02 - 2	0.9911
16OH-DHP4	2.89	333.20 > 145.10	333.20 > 159.10	333.20 > 213.20	30	30	30	15	15	15	0.02 - 2	-
11,16diOHP4	1.78	347.22 > 121.06	347.22 > 269.20	347.22 > 311.20	30	30	30	20	20	15	0.02 - 2	-
11,16diOH-DHP4	1.87	349.20 > 97.10	349.20 > 313.40	-		15	15	25	20		0.02 - 2	-
3,16diOH-DHP4	3.09	335.20 > 104.60	335.20 > 118.70	335.20 > 145.20	30	30	30	35	40	40	0.02 - 2	-

Supplementary table 3

UHPLC-MS/MS gradient applied in the separation of steroids listed in supplementary table 4. Injection volume was set to 5 µL, flow rate 0.4 mL/min.

	Time (min)	**%A**	**%B**	**curve**
1	initial	85.0	15.0	Initial
2	0.50	60.0	40.0	6
3	3.50	35.0	55.0	6
4	3.70	0.0	68.0	6
5	3.71	0.0	100.0	6
6	4.00	85.0	100.0	6
7	4.01	85.0	15.0	6
8	5.00	85.0	15.0	6

Supplementary table 4

UHPLC-MS/MS separation and quantification of C_{19} and C_{21} steroids. The gradient described in supplementary table 3 was used to separate the steroids tabulated below. Each steroid is presented with its retention time (RT), Molecular (parent) and product (daughter) ion species together with the respective mass spectrometry properties: such as the cone voltage (CV), collision energy (CE), calibration range (ng/µL) and the linearity (r^2). The limit of quantification (LOQ) is 0.02 ng/µL.

Steroid	RT (min)	Mass transitions		Cone Voltage (V)		Collision Energy (eV)		Calibration range (ng/µL)	Linearity (r^2)
		Product ion 1	Product ion 2						
Internal standards									
D2-T	3.68	291.00 > 99.10	291.00 > 111.25	30	30	20	20	-	-
D7-11OHA4	2.59	310.20 > 147.20	310.20 > 99.80	25	25	25	30	-	-
D7-A4	3.78	294.30 > 100.00	294.30 > 113.00	25	25	25	25	-	-
D8-17OHP4	4.14	340.10 > 100.00	340.10 > 114.00	26	26	25	28	-	-
D9-P4	4.37	324.20 > 100.00	324.20 > 113.00	30	30	20	25	-	-
D4-Cortisol	2.02	367.00 > 121.00		35		25		-	-
C_{19} steroids									
A4	3.81	287.20 > 96.90	287.20 > 108.80	30	30	15	15	0.02-2	0.9965
11OHA4	2.62	303.20 > 267.20	303.20 > 121.20	30	30	15	30	0.02-2	0.9883
T	3.69	289.20 > 97.20	289.20 > 109.00	30	30	22	22	0.02-2	0.9967
11OHT	2.46	305.30 > 121.00	305.30 > 269.00	35	35	20	15	0.02-2	0.9833
C_{21} steroids									
P4	4.38	315.20 > 96.90	315.20 > 297.20	30	30	15	15	0.02-2	0.9924
17OHP4	4.15	331.10 > 97.00	331.10 > 108.90	30	30	15	15	0.02-2	0.9811
16OHP4	2.98	331.20 > 97.00	331.20 > 108.90	30	30	15	15	0.02-2	0.9899

11,16diOHP4	1.63	347.22 > 121.06	347.22 > 311.20	30	30	25	15	0.02-2	-
11OHP4	3.97	331.20 > 121.00	331.20 > 295.20	30	30	20	20	0.02-2	0.9944
21dF	2.65	347.10 > 121.00	347.10 > 269.20	25	25	20	20	0.02-2	0.9873
CORT	2.80	347.00 > 121.00	347.00 > 329.10	30	30	15	15	0.02-2	0.9981
DOC	3.86	331.20 > 97.00	331.20 > 108.90	30	30	15	15	0.02-2	0.9944
ALDO	1.68	361.40 > 97.00	361.40 > 343.20	30	30	32	18	0.02-2	0.9944
Deoxycortisol	2.89	347.00 > 97.00	347.00 > 108.90	30	30	15	15	0.02-2	0.9863
Cortisol	2.03	63.00 > 121.00		30		20		0.02-2	0.9973

Supplementary table 5

UHPLC-MS/MS gradient applied in the separation of steroids listed in supplementary table 6. Injection volume was set to 5 µL, and the flow rate 0.4 mL/min.

	Time (min)	**%A**	**%B**	**curve**
1	initial	85.0	15.0	Initial
2	0.04	70.0	30.0	6
3	3.12	65.0	35.0	5
4	4.67	62.0	38.0	6
5	5.45	61.0	39.0	6
6	7.10	40.0	60.0	6
7	7.20	0.0	100.0	6
8	7.70	0.0	100.0	6
9	7.80	85.0	100.0	6
10	10.00	85.0	100.0	6

Supplementary table 6

UHPLC-MS/MS separation and quantification of C_{19} and C_{21} steroids. The gradient described in supplementary table 5 was used to separate the steroids tabulated below. Each steroid is presented with its retention time (RT), molecular (parent) and product (daughter) ion species together with the respective mass spectrometry properties such as the cone voltage (CV), collision energy (CE), calibration range (ng/µL) and the linearity (r^2). The limit of quantification (LOQ) is 0.02 ng/µL.

Steroid	RT (min)	Mass transitions		Cone Voltage (V)		Collision Energy (eV)		Calibration range (ng/µL)	Linearity (r^2)
		Product ion 1	Product ion 2						
Internal standards									
D2-T	4.23	291.00 > 99.10	291.00 > 111.25	25	25	30	30	-	-
D9-P4	7.13	324.20 > 100.00	324.20 > 113.00	30	30	20	25	-	-
C_{19} steroids									
A4	4.57	287.20 > 96.90	287.20 > 108.80	30	30	15	15	0.02-2	0.9961
11KA4	2.66	301.20 > 257.00	301.20 > 241.20	35	35	30	25	0.02-2	0.9986
AST	6.15	273.20 > 105.30	291.30 > 147.00	30	30	30	25	0.02-2	0.9796
11KAST	3.11	305.00 > 147.00	305.00 > 107.00	15	15	30	25	0.02-2	0.9889
C_{21} steroids									
P4	7.13	315.20 > 96.90	315.20 > 297.20	30	30	15	15	0.02-2	0.9872
17OHP4	5.07	331.10 > 97.00	331.10 > 108.90	30	30	15	15	0.02-2	0.9932
Pdiol	6.83	317.40 > 111.00	317.40 > 299.00	15	15	20	15	0.02-2	0.9723
Alfaxalone	4.84	333.20 > 147.20	333.20 > 159.27	25	25	30	30	0.02-2	0.9718
21dE	3.05	345.20 > 162.80	345.20 > 121.10	25	25	25	20	0.02-2	0.9899

Supplementary table 7

UPC^2-MS/MS gradient applied in the separation of steroids listed in supplementary table 8. Injection volume was set to 2 µL, and the flow rate 2 mL/min.

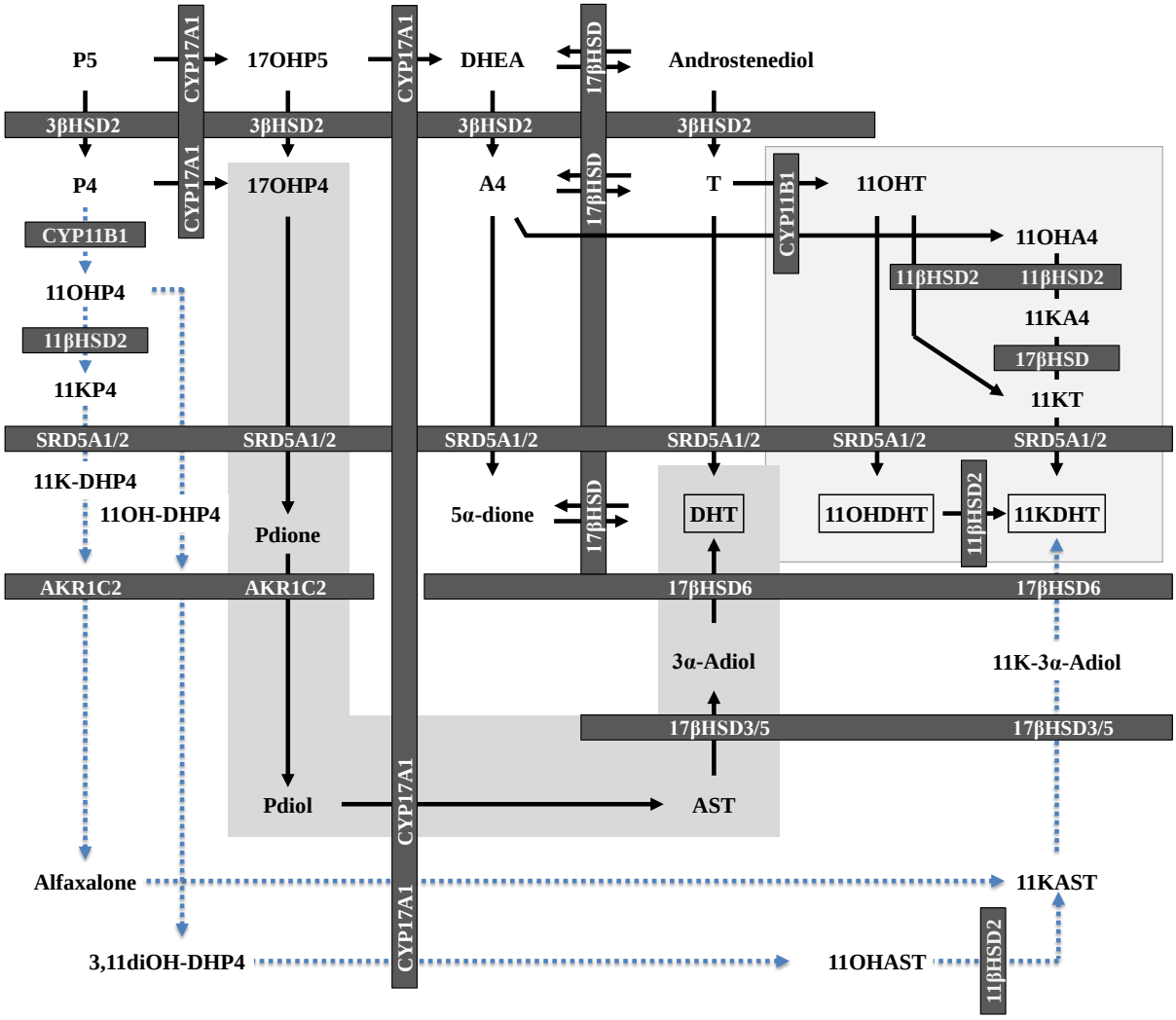
	Time (min)	**%A**	**%B**	**curve**
1	initial	98.0	2.0	Initial
2	2.70	90.0	10.0	7
3	3.80	88.0	12.0	6
4	4.30	82.0	18.0	6
5	5.40	75.0	25.0	6
6	5.50	98.0	2.0	1
7	6.50	98.0	2.0	1

Supplementary table 8

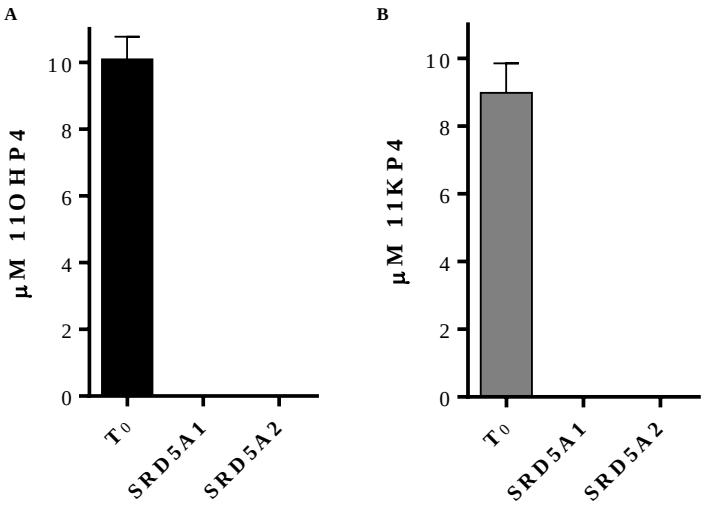
UPC^2-MS/MS separation and quantification of C_{19} and C_{21} steroids. The gradient described in supplementary table 7 was used to separate the steroids listed. Each steroid is presented with its retention time (RT), molecular (parent) and daughter ion species together with the respective mass spectrometry properties such as the cone voltage (CV), collision energy (CE), calibration range (ng/µL) and the linearity (r^2). The limit of quantification (LOQ) of 11OHAST and 11KAST is 0.01 ng/µL and all other steroids 0.001 ng/µL.

Steroid	RT (min)	Mass transitions		Cone Voltage (V)		Collision Energy (eV)		Calibration range (ng/µL)	Linearity (r^2)
		Quantifier	Qualifier						
Internal standards									
D2-T	2.91	291.00 > 99.10	291.00 > 111.25	30	30	20	20	-	-
D7-11OHA4	3.05	310.20 > 147.20	310.20 > 99.80	25	25	25	30	-	-
D7-A4	1.82	294.30 > 100.00	294.30 > 113.00	25	25	25	25	-	-
D8-17OHP4	2.71	340.10 > 100.00	340.10 > 114.00	26	26	25	28	-	-
D9-P4	1.68	324.20 > 100.00	324.20 > 113.00	30	30	20	25	-	-
C_{19} steroids									
A4*	1.83	287.20 > 96.90	287.20 > 108.80	30	30	15	15	0.001-1	0.9976
11OHA4*	3.05	303.20 > 267.20	303.20 > 121.20	30	30	15	30	0.001-1	0.9992
11KA4*	2.43	301.20 > 257.00	301.20 > 265.20	35	35	25	25	0.001-1	0.9991
T*	2.91	289.20 > 97.20	289.20 > 109.00	30	30	22	22	0.001-1	0.9981
11OHT*	4.25	305.30 > 121.00	305.30 > 269.00	35	35	20	15	0.001-1	0.9994
11KT*	3.57	303.20 > 121.00	303.20 > 267.00	30	30	20	20	0.001-1	0.9988
5α-dione*	1.11	289.18 > 253.14	289.20 > 97.20	22	30	16	22	0.001-1	0.9964
3α-Adiol*	3.27	275.20 > 257.00	275.20 > 175.00	15	15	15	15	0.001-1	0.9982
DHEA	2.30	271.21 > 253.2	271.21 > 243	30	30	15	15	0.001-1	0.9958

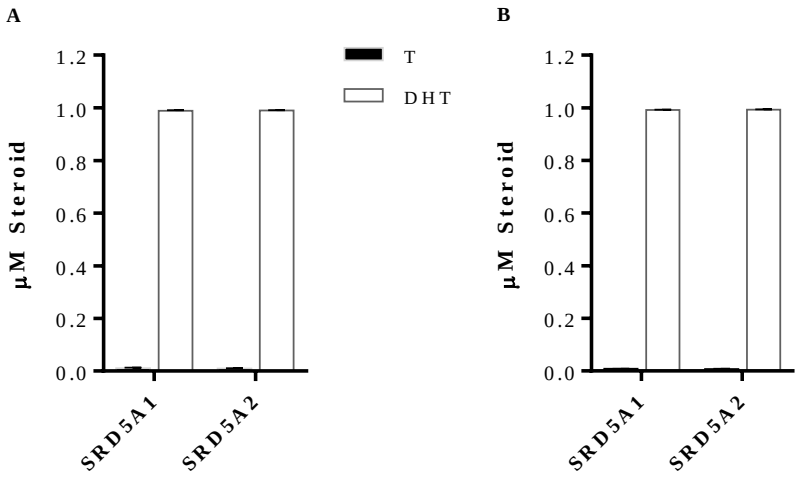
DHT*	2.20	291.20 > 255.00	291.20 > 273.00	25	25	15	20	0.001-1	0.9960
11KDHT*	3.22	305.20 > 243.00	305.20 > 269.00	30	30	20	20	0.001-1	0.9996
AST*	2.24	273.20 > 105.30	291.30 > 273.30	30	18	30	8	0.001-1	0.9971
11OHAST*	3.44	289.00 > 271.00	289.00 > 213.00	15	15	15	15	0.01-1	0.9988
11KAST*	3.01	305.00 > 147.20	305.00 > 173.10	30	30	30	30	0.01-1	0.9992
C_{21} steroids									
P4	1.68	315.20 > 97.00	315.20 > 109.00	28	28	20	26	0.001-1	0.9947
DHP4	0.98	317.00 > 105.20	317.00 > 95.00	30	30	30	30	0.001-1	0.9978
17OHP4	2.72	331.10 > 97.00	331.10 > 109.00	26	26	22	28	0.001-1	0.9982
Pdiol	3.32	317.40 > 111.00	317.40 > 299.00	20	20	25	15	0.001-1	0.9898
Pdione	2.03	333.40 > 159.00	333.40 > 137.00	20	20	25	25	0.001-1	0.9953
Pregnanetriol	4.20	301.20 > 135.00	301.20 > 81.00	25	25	15	25	0.001-1	0.9915
16OHP4	3.41	331.20 > 97.00	331.20 > 108.90	30	30	15	15	0.001-1	0.9939
11OHP4	3.22	331.20 > 121.00	331.20 > 295.00	30	30	20	20	0.001-1	0.9943
11αOHP4	3.53	331.20 > 295.20	331.20 > 121.00	30	30	15	30	0.001-1	0.9909
11KP4	2.45	329.21 > 121.00	329.21 > 84.80	15	15	20	20	0.001-1	0.9920
11K-DHP4	1.91	331.20 > 105.00	331.20 > 147.00	25	25	30	30	0.001-1	0.9981
Alfaxalone	3.20	333.20 > 147.27	333.20 > 159.27	25	25	30	30	0.001-1	0.9856
21dE	3.17	345.20 > 162.80	345.20 > 121.10	25	25	25	20	0.001-1	0.9980
21dF	3.80	347.10 > 121.00	347.10 > 269.20	20	20	25	15	0.001-1	0.9963



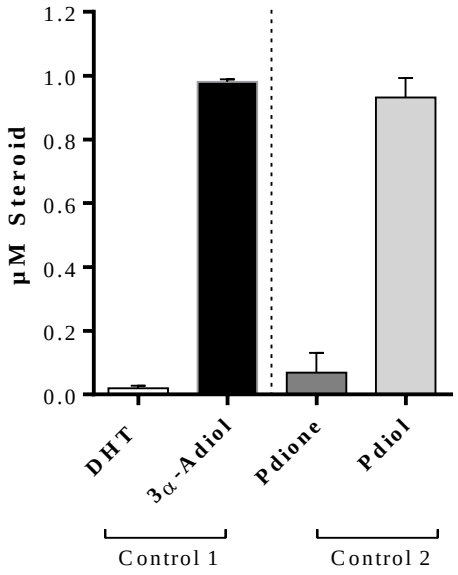
Supplementary figure 1 11β-hydroxyprogesterone (11OHP4) and 11-ketoprogesterone (11KP4) are converted to 11-ketodihydrotestosterone (11KDHT) by steroidogenic enzymes in the backdoor pathway. The metabolism of 11OHP4 to 11KDHT: dotted lines; the metabolism of 17α-hydroxyprogesterone (17OHP4) to dihydrotestosterone (DHT): shaded dark grey; and the metabolism of 11β-hydroxyandrostenedione (11OHA4) and 11β-hydroxytestosterone (11OHT) to 11β-hydroxydihydrotestosterone (11OHDHT) and 11KDHT: shaded light grey.



Supplementary figure 2: SRD5A isoforms catalyse the 5α-reduction of C11-oxy C_{21} steroids. Conversion of 10 µM (A) 11β-hydroxyprogesterone (11OHP4) and (B) 11-ketoprogesterone (11KP4) by SRD5A1 and SRD5A2 in HEK-293. Results are expressed as the mean ±SD of an independent experiment performed in triplicate (n=3).



Supplementary figure 3: SRD5A isoforms catalyse the 5α-reduction of T to DHT. SRD5A was expressed in HEK-293 cells and the substrate, testosterone (T) and product, dihydrotestosterone (DHT) analysed after 12 hrs.



Supplementary figure 4: AKR1C2 catalyses the 3α-reduction of DHT to 3α-Adiol and Pdione to Pdiol. AKR1C2 was expressed in HEK-293 cells and the substrates (dihydrotestosterone (DHT) and 5α-pregnan-17α-ol-3,20-dione (Pdione)) and products (5α-androstane-3α,17β-diol (3α-Adiol) and 5α-pregnan-3α,17α-diol-20-one (Pdiol)) analysed after 12 hrs.

Declaration by the candidate:

With regard to Chapter 3, pages 46-68, the nature and scope of my contribution were as follows:

Nature of contribution: Desmaré van Rooyen completed the following: generated the bulk of the data reported in the article and all the data in the supplemental file; identified and characterised steroid metabolites using Q-TOF-MS and developed the UHPLC-MS/MS and UPC^2-MS/MS methods, analysed and processed all the data; complied all tables and figures, the graphical abstract and the steroid schematic (Figure 1); compiled the first draft of the manuscript, researched articles relevant to manuscript used in the introduction and discussion.

Extent of contribution: data, 85%; manuscript first draft 100%; joint editing of manuscript (50%) with AC Swart.

The following co-authors have contributed to Chapter 3, pages 46-68:

Name; e-mail address; nature of contribution; extent of contribution (%)

1. Rachelle Gent: rgent@sun.ac.za; generated data pertaining to 11βHSD conversion shown in Figure 4, contributing 5 % of all reported data. Read the final manuscript draft.
2. Lise Barnard: liseb@asun.ac.za; generated a replicate data set for the P4 conversion by CYP11B2 shown in Figure 3, contributing 10 % of all reported data. Read the final manuscript draft.
3. Amanda C. Swart; acswart@sun.ac.za; principal investigator and corresponding senior author.

Signature of candidate: *_____

Date: 8 January 2020

Declaration by co-authors:

The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapter 3, pages 46-68,
2. no other authors contributed to Chapter 3, pages 46-68, besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapter 3, pages 46-68, of this dissertation.

Signature	Institutional affiliation	Date
*_____	Stellenbosch University	8 January 2020
*_____	Stellenbosch University	8 January 2020
*_____	Stellenbosch University	8 January 2020

*Declaration with signature in possession of candidate and supervisor.

Chapter 4

Published manuscript: CYP17A1 exhibits 17α-hydroxylase/17,20-lyase activity towards 11β-hydroxyprogesterone and 11-ketoprogesterone metabolites, in the C11-oxy backdoor pathway.

4.1 Introduction

Cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) is a microsomal enzyme with a dual-enzymatic function involved in the biosynthesis of glucocorticoids and androgens. CYP17A1 catalyses the 17α-hydroxylation of pregnenolone (P5) and progesterone (P4) and 17,20-carbon-carbon cleavage (17,20-lyase) of 17α-hydroxypregnenolone (17OHP5); however, the substrate specificity of CYP17A1 with regards to the hydroxylase and lyase activity is very selective. In adrenal steroidogenesis, P5 is hydroxylated and subsequently lyased to 17OHP5 and high levels of dehydroepiandrosterone (DHEA), respectively, in the Δ^5-pathway; whereas P4 —the 3βHSD2-catalysed P5 metabolite, is hydroxylated to 17α-hydroxyprogesterone (17OHP4) in the Δ^4-pathway with negligible androstenedione (A4) formation in humans. In addition, human and primate CYP17A1 possess the unique ability to hydroxylate P4 at C16 (Swart *et al.* 1993). In the adrenal, the 17,20-lyase reaction favours 17OHP5 ($_{app}K_m$= 1.2 µM) over 17OHP4 regardless of their similar binding affinities (Flück *et al.* 2003, Petrunak *et al.* 2014). The above-mentioned places emphasise on the regulatory role of the lyase activity of CYP17A1 determining the flux not only through the glucocorticoid pathway but also through the different androgen pathways (Kamrath *et al.* 2012) —influenced by tissue expression, substrate affinity and presence or absence of co-factors and/or co-enzymes including cytochrome b_5 (cyt b_5). In the backdoor pathway, CYP17A1 rapidly lyases the 5α, 3α-reduced 17OHP4 metabolite, 5α-pregnan-3α,17α-diol-20-one (Pdiol) to androsterone (AST) with an apparent K_m (0.6 µM) significantly higher than that of the enzyme for the natural substrate, 17OHP5, even in the absence of cyt b_5 (Gupta *et al.* 2003).

In Chapter 3, we showed the metabolism of 11OHP4 and 11KP4 by the steroidogenic enzymes in the backdoor pathway and the conversion of 11KP4 and its metabolites, including alfaxalone to 11KAST, catalysed by CYP17A1; however, the structural interaction and catalytic activity of CYP17A1 towards the C11-oxy C_{21} steroids remain largely unexplored despite the association of the C11-oxy C_{21} steroids with elevated 17OHP4 levels.

CYP17A1 is expressed in all the major steroidogenic tissue including ovarian theca cells (Marti *et al.* 2017), adrenal zona fasciculata and reticularis, leydig cells, as well as prostate cancer tumours and cell models such as the androgen-dependent 22RV1 cell model (Giatromanolaki *et al.* 2019). CYP17A1 plays a crucial role in androgen-dependent and independent cancers including prostate cancer (PCa) and castration-resistant prostate cancer (CRPC), the latter associated with increased backdoor pathway activity, with the CYP17A1 expression closely related to androgen receptor expression (Giatromanolaki *et al.* 2019). Elevated T and DHT levels were detected in tissue of CRPC compared to post-castration tissue which did not reflect in the corresponding serum (Locke *et al.* 2008). This addresses a limitation for serum steroid profiles and that conclusions based on serum, urine, hair or saliva samples would not necessarily reflect the intratissue steroid profile. Reports of the transactivation of the androgen receptor by low levels of DHT (nM range) (Gregory *et al.* 2001, Storbeck *et al.* 2013) emphasise the importance of considering all potential pathways which may contribute to the androgen pool. In addition, elevated CYP17A1 expression in the presence of C11-oxy steroids have also been reported in hyperandrogenic disorders such as polycystic ovary syndrome (PCOS) (Comim *et al.* 2013, Marti *et al.* 2017) and 21-hydroxylase deficiency (21OHD) patience with testicular adrenal rest tumours in addition to elevated mRNA levels of CYP11B1 and CYP11B2 (Smeets *et al.* 2015). The prevalence in the aforementioned condition, in tissue and the broad but intricate substrate specificity of CYP17A1, emphasises the importance of investigating CYP17A1's activity towards the C11-oxy C_{21} steroids and the potential contribution of the metabolic products to the androgen pool.

In this chapter, we therefore aimed to elucidate the catalytic activity of CYP17A1 towards the C11-oxy C_{21} steroids as well as substrate interaction with amino acid residues in the active site of the enzyme. Once custom-made C11-oxy C_{21} steroids were available, the steroids were characterised for mass spectrometry analysis and an UPC^2-MS/MS method was developed to quantify C11-oxy C_{19} and C11-oxy C_{21} steroids. We explored the substrate specificity of human CYP17A1 in terms of the 17α-hydroxylase and 17,20-lyase reactions towards the C11-oxy C_{21} steroids. These C11-oxy C_{21} steroids and their downstream metabolites were thus assayed in transiently transfected HEK-293 cells. The CYP17A1 lyase conversion of cortisol and 5α/5β-tetrahydrocortisol to C11-oxy C_{19} steroids was also investigated since these reactions have been suggested to occur *in vivo*. In addition, we report the docking of 11OHP4, 11KP4 and their 5α-reduced metabolites in the active site of human CYP17A1. The approach followed in docking

the C11-oxy C_{21} steroids in the active site had certain limitations which should be acknowledged. Modelling CYP17A1 with P4 or P5 in the active site as a starting point prior to docking the C11-oxy C_{21} steroids would constrain potential structures. In addition, back mutating L105A in the CYP17A1 structure can potentially lead to inaccurate assumptions regarding the long-range cooperative changes in the cytochrome P450 structure.

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CYP17A1 exhibits 17αhydroxylase/17,20-lyase activity towards 11β-hydroxyprogesterone and 11-ketoprogesterone metabolites in the C11-oxy backdoor pathway

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11β-hydroxyetiocholanolone (11βOHETIO)
11β-hydroxysteroid dehydrogenase (11βHSD, HSD11B)

ABSTRACT

Cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) plays a pivotal role in the regulation of adrenal and gonadal steroid hormone biosynthesis. More recent studies highlighted the enzyme's role in the backdoor pathway leading to androgen production. Increased CYP17A1 activity in endocrine disorders and diseases are associated with elevated C_{21} and C_{19} steroids which include 17α-hydroxyprogesterone and androgens, as well as C11-oxy C_{21} and C11-oxy C_{19} steroids. We previously reported that 11β-hydroxyprogesterone (11OHP4), 21-deoxycortisol (21dF) and their keto derivatives are converted by 5α-reductases and hydroxysteroid dehydrogenases yielding C_{19} steroids in the backdoor pathway. In this study the 17α-hydroxylase and 17,20-lyase activity of CYP17A1 towards the unconventional C11-oxy C_{21} steroid substrates and their 5α- and 3α,5α-reduced metabolites was investigated in transfected HEK-293 cells. CYP17A1 catalysed the 17α-hydroxylation of 11OHP4 to 21dF and 11-ketoprogesterone (11KP4) to 21-deoxycortisone (21dE) with negligible hydroxylation of their 5α-reduced metabolites while no lyase activity was detected. The 3α,5α-reduced C11-oxy C_{21} steroids—5α-pregnan-3α,11β-diol-20-one (3,11diOH-DHP4) and 5α-pregnan-3α-ol-11,20-dione (alfaxalone) were rapidly hydroxylated to 5α-pregnan-3α,11β,17α-triol-20-one (11OH-Pdiol) and 5α-pregnan-3α,17α-diol-11,20-dione (11K-Pdiol), with the lyase activity subsequently catalysing to conversion to the C11-oxy C_{19} steroids, 11β-hydroxyandrosterone and 11-ketoandrosterone, respectively. Docking of 11OHP4, 11KP4 and the 5α-reduced metabolites, 5α-pregnan-11β-ol-3,20-dione (11OH-DHP4) and 5α-pregnan-3,11,20-trione (11K-DHP4) with human CYP17A1 showed minimal changes in the orientation of these C11-oxy C_{21} steroids in the active pocket when compared with the binding of progesterone suggesting the 17,20-lyase is impaired by the C11-hydroxyl and keto moieties. The structurally similar 3,11diOH-DHP4 and alfaxalone showed a greater distance between C17 and the heme group compared to the natural substrate, 17α-hydroxypregnenolone potentially allowing more orientational freedom and facilitating the conversion of the C11-oxy C_{21} to C11-oxy C_{19} steroids.

In summary, our *in vitro* assays showed that while CYP17A1 readily hydroxylated 11OHP4 and 11KP4, the enzyme was unable to catalyse the 17,20-lyase reaction of these C11-oxy C_{21} steroid products. Although CYP17A1 exhibited no catalytic activity towards the 5α-reduced intermediates, once the C4-C5 double bond and the keto group at C3 were reduced, both the hydroxylation and lyase reactions proceeded efficiently. These

Abbreviations: P4, progesterone; P5, pregnenolone; DHP4, dihydroprogesterone or 5α-pregnan-3,20-dione; 16OHP4, 16α-hydroxyprogesterone; 17OHP4, 17α-hydroxyprogesterone; Pdione, 5α-pregnan-17α-ol-3,20-dione; Pdiol, 5α-pregnan-3α,17α-diol-20-one; 11OHP4, 11β-hydroxyprogesterone; 11KP4, 11-ketoprogesterone; 11OH-DHP4, 5α-pregnan-11β-ol-3,20-dione; 11K-DHP4, 5α-pregnan-3,11,20-trione; 3,11diOH-DHP4, 5α-pregnan-3α,11β-diol-20-one; alfaxalone, 5α-pregnan-3α-ol-11,20-dione; 21dF, 4-pregnen-11β,17α-diol-3,20-dione or 21-deoxycortisol; 21dE, 4-pregnen-17α-ol-3,11,20-trione or 21-deoxycortisone; 11OH-Pdione, 5α-pregnan-11β,17α-diol-3,20-dione; 11K-Pdione, 5α-pregnan-17α-ol-3,11,20-trione; 11OH-Pdiol, 5α-pregnan-3α,11β,17α-triol-20-one; 11K-Pdiol, 5α-pregnan-3α, 17α-diol-11,20-dione; DHEA, dehydroepiandrosterone; 3αAdiol, 5α-androstane-3α,17β-diol; 11OH-3αAdiol, 5α-androstane-3α,11β,17β-triol; 11K-3αAdiol, 5α-androstane-3α,17β-diol-11-one; 5αDione, 5α-androstanedione; 11OH-5αDione, 11β-hydroxy-5α-androstanedione; 11K-5αDione, 11-keto-5α-androstanedione; A4, androstenedione; 11OHA4, 11β-hydroxyandrostenedione; 11KA4, 11-ketoandrostenedione; T, testosterone; 11OHT, 11β-hydroxytestosterone; 11KT, 11-ketotestosterone; DHT, dihydrotestosterone; 11OHDHT, 11β-hydroxydihydrotestosterone; 11KDHT, 11-ketodihydrotestosterone; AST, androsterone; 11OHAST, 11β-hydroxyandrosterone; 11KAST, 11-ketoandrosterone; 11OH-etiocholanolone or 11OH-etio, 5β-androstan-3α,11β-diol-17-one

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findings show that the C11-oxy C_{21} steroids could potentially contribute to the androgen pool in tissue expressing steroidogenic enzymes in the backdoor pathway.

1. Introduction

The past two decades have seen an increase in studies reporting elevated levels of C11-oxy C_{19} and C11-oxy C_{21} steroids, 17α-hydroxyprogesterone (17OHP4) and androgens in pathophysiological conditions such as congenital adrenal hyperplasia, 21-hydroxylase deficiency (21OHD) [1–5], prostate cancer (PCa) [6,7], benign prostatic hyperplasia (BPH) [8] and polycystic ovary syndrome (PCOS) [9–11].

The biosynthesis of 17OHP4 and androgens rely on cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1), a microsomal steroidogenic enzyme in the adrenal cortex, testis [12] and ovary [13], that plays a crucial role in maintaining steroidogenic homeostasis. In adrenal steroidogenesis, CYP17A1 catalyses the 17α-hydroxylation of pregnenolone (P5) and progesterone (P4) to 17α-hydroxypregnenolone (17OHP5) and 17OHP4, respectively. While the 17OHP4 provides the precursor for glucocorticoid production leading to cortisol formation, the 17,20-lyase activity of CYP17A1 converts 17OHP5 to dehydroepiandrosterone (DHEA). Cleavage of the side chain, which converts the C_{21} steroid to the C_{19} steroid, is enhanced by the allosteric interaction of cytochrome b_5 (cyt b_5) [14], accounting for the production of adrenal androgens via the Δ^5-pathway. In contrast, the conversion of 17OHP4 to androstenedione (A4) in the Δ^4pathway is negligible in humans. In human and chimpanzee CYP17A1, the Ala residue at 105 of the β-helix allows more movement of the substrate in the active site resulting in the hydroxylation at C16 of P4 but not of pregnenolone (P5) due to the difference in orientation of the Δ^5-3β-hydroxy and Δ^4-3-keto steroids in the active pocket [15,16].

Male and female gonadal steroidogenic pathways also depend on CYP17A1 activity. Similar to the adrenal androgen pathway, leydig cells in the testis convert cholesterol to testosterone (T) via the CYP17A1-catalysed conversion of P5 with the androgen precursor dehydroepiandrosterone (DHEA) as an intermediate. In females, ovarian theca cells express high levels of CYP17A1 which converts the *de novo* biosynthesised P4 to 17OHP4 and androgens which subsequently diffuse to granulosa cells for estradiol biosynthesis [17].

More recently, our steroidogenic research has focused on the metabolism of the C11-oxy C_{21} steroids in backdoor pathway yielding potent androgens (Fig. 1). Increased activation of the pathway and elevated enzyme expression levels are more frequently reported in the human fetus [18–20] and ovaries [11] and in clinical conditions which include 21OHD [3,21] and adrenocortical tumours [22].

CYP17A1 in the backdoor pathway has been reported to efficiently catalyse the side-chain cleavage of 5α-pregnan-3α,17α-diol-20-one (Pdiol)—the 3α,5α-reduced metabolite of 17OHP4, to androsterone (AST) with an activity higher than exhibited towards the natural substrate, 17α-hydroxypregnenolone (17OHP5) [21,23]. Unlike the 13-fold increase in lyase activity by cyt b_5 towards 17OHP5 [14], Pdiol is rapidly converted to AST, in the absence of cyt b_5, in the backdoor pathway with only a 3-fold increase when cyt b_5 is co-expressed [23].

The detection of C11-oxy steroids in clinical conditions associated with increased backdoor pathway activity led us to investigate the catalytic

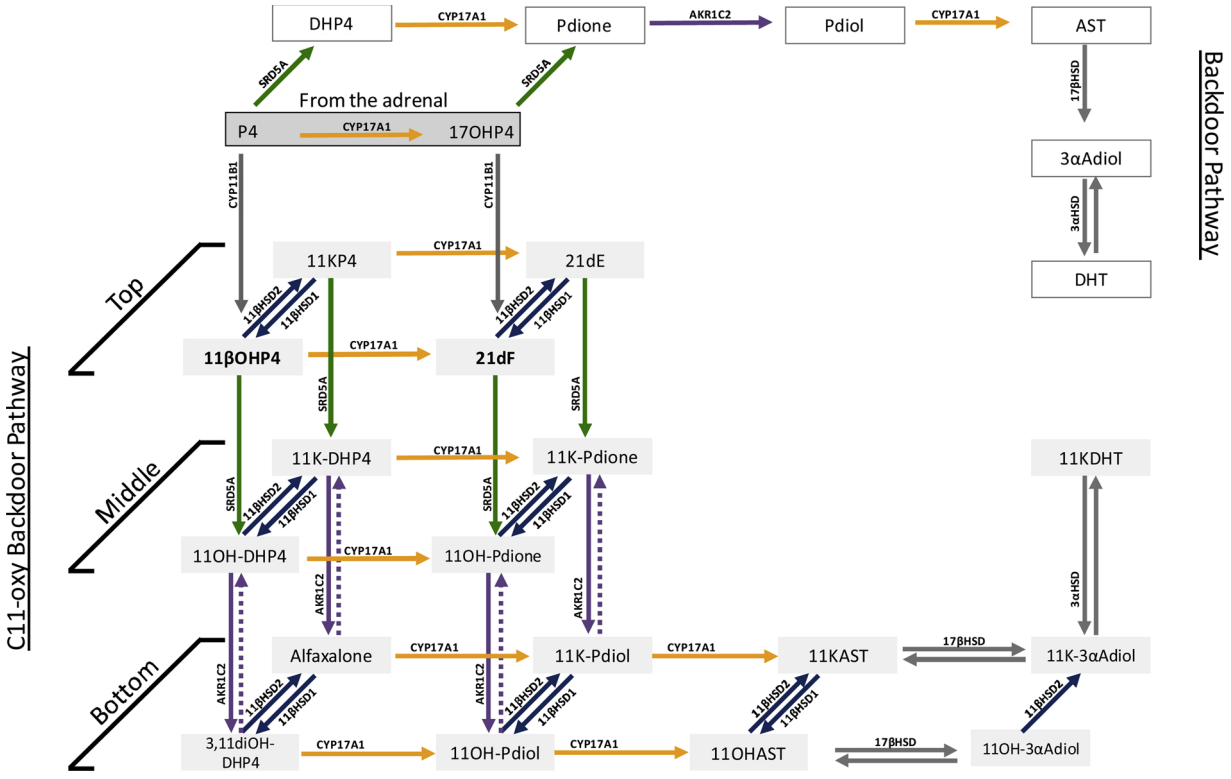

Fig. 1. Metabolism of adrenal C11-oxy progesterones in the backdoor pathway. 17α-Hydroxyprogesterone (17OHP4) is metabolised to dihydrotestosterone (DHT) while 11β-hydroxyprogesterone (11OHP4), 11-ketoprogesterone (11KP4), 21-deoxycortisol (21dF) and 21-deoxycortisone (21dE) are metabolised to 11KDHT. Steroidogenic enzymes catalyse the production of C_{19} steroids and C11-oxy C_{19} steroids in the backdoor pathways: cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1), yellow arrows; 5α-reductase (SRD5A), green arrows; aldo-keto reductase (AKR1C2), purple arrows; 17β-hydroxysteroid dehydrogenases (17βHSD) and 3α hydroxysteroid dehydrogenase (3αHSD), grey arrows. Cytochrome P450 11β-hydroxylase (CYP11B1), grey arrows, catalyses the biosynthesis of C11-oxy progesterones and 11β-hydroxysteroid dehydrogenases (11βHSD1 and 11βHSD2), navy arrows, catalyse the interconversion of the C11 keto/hydroxyl steroid intermediates. Dashed lines represent potential conversions not shown in vitro.

activity of CYP17A1 towards the C11-oxy C_{21} steroids potentially contributing to the androgen pool. We addressed the biological origin of C11-oxy C_{21} steroids in an *in vitro* study reporting the hydroxylation of P4 and 17OHP4 at C11 by adrenal cytochrome P450 11β-hydroxylase (CYP11B1) and cytochrome P450 aldosterone synthase (CYP11B2) yielding 11β-hydroxyprogesterone (11OHP4) and 21-deoxycortisol (21dF), respectively, and the subsequent oxidation to their keto derivative, 11-ketoprogesterone (11KP4) and 21-deoxycortisone (21dE) catalysed by 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2). Furthermore, our investigations demonstrated that the reduction of the C4-C5 double bond and the keto moiety at C3 of 11OHP4 and 21dF as well as that of their C11-keto derivatives to the respective 3α,5α-reduced metabolites by steroidogenic enzymes are characteristic of the backdoor pathway (Fig. 1). The rapid 5α-reduction of C11-oxy C_{21} steroids by the backdoor pathway gatekeeper, 5α-reductase (SRD5A), showed substrate superiority over T and 17OHP4 [24,25].

Backdoor pathway activity in 21OHD is further supported by the markedly higher Pdiol, AST and 11β-hydroxyandrosterone (11OHAST) levels with a 40 and 60-fold increase in 11OHAST/Δ^5 metabolites in infants and neonates, respectively [3]. Furthermore, Kamrath et al. reported high 5α-reductase- and backdoor pathway activities towards androgen production in which 11OHAST and etiocholanolone represented the classical androgen pathways by analysing steroid ratios [3,21]. Unfortunately, these reports did not consider the potential contribution of the C11-oxy C_{21} steroids metabolised via the C11-oxy backdoor pathway to 11OHAST and 11-ketoandrosterone (11KAST).

In this study, the *in vitro* 17α-hydroxylase and 17,20-lyase activity of human CYP17A1 towards the C11-oxy C_{21} steroid metabolites, including cortisol and its metabolites, and their contribution to the C11-oxy C_{19} steroid pool was investigated. Considering the effect of the orientational difference of P5 (a Δ^5-3β-hydroxy steroid) and P4 (a Δ^4-3-keto steroid) in the human CYP17A1 active site on the catalytic capability, and the enzyme's low apparent K_m for Pdiol, we investigated whether the presence of the C11-oxy group hinders the docking and binding of 11OHP4, 11KP4 and their respective 5α- and 3α,5α-reduced metabolites in the enzyme's active site.

2. Materials and methods

2.1. Materials

Methyl *tert*-butyl ether (MTBE), Dulbecco's Modified Eagle's Medium (DMEM), D-(+)-Glucose and 4-pregnene-11β, 17α,21-triol-3,20-dione (cortisol), dihydroprogesterone (DHP4), 17OHP4 and A4 were purchased from Sigma-Aldrich (St. Louis, USA). Human embryonic kidney cells (HEK-293) cells were purchased from the American Type Culture Collection, ATCC (Manassas, USA). Corning® CellBIND® surface tissue cultureware (T75 flasks, 100 mm dishes, and 24-well plates) were purchased from Corning® Life Science (NY, USA). Trypan blue (0.4 %) stain and Countess® cell count plates were purchased from Invitrogen (Eugene, USA). X-tremeGENE HP® DNA transfection reagent was obtained from Roche Diagnostics (Mannheim, Germany). Trypsin-EDTA, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Oxoid limited (Hampshire, England). The Promega Wizard® Plus Midipreps DNA purification system (Madison, USA) was purchased from Anatech. 4-androstene-11β-ol-3,17-dione (11OHA4), 4-androsten-3,11,17-trione (11KA4), 5α-androstan-3,17-dione (5αDione), 5α-androstan-11β-ol-3,17-dione (11OH-5αDione), 5α-androstan-3,11,17-trione (11K-5αDione), 5α-androstan-17β-ol-3,11-dione (11KDHT), 5α-pregnan-11α-ol-3,20-dione (11αOH-DHP4), 4-pregnen-11α-ol-3,20-dione (11αOHP4), 4-androsten-11β,17β-diol-3-one (11OHT), 4-androsten-17β-ol-3,11-dione (11KT), 5α-androstan-3α-ol-17-one (AST), 5α-androstan-3α,11β-diol-17-one (11OHAST), 5α-androstan-3α-ol-11,17-dione (11KAST), 11OH-etiocholanolone (11OH-etio), 5α-pregnan-17α-ol-3,20-dione (Pdione), 5α-pregnan-3α,17α-diol-20-one (Pdiol), 3α,11β,17α,21-tertahydroxy-5β-pregnan-20-one (tetrahydrocortisol), 4-pregnen-11β-ol-3,20-dione (11βOHP4), 4-pregnen-3,11,20-trione (11KP4), 5α-pregnan-3,11,20-trione (11K-DHP4), 5α-pregnan-3α-ol-11,20-dione (alfaxalone), 4-pregnen-11β,17-diol-3,20-dione (21dF), 4-pregnen-17-ol-3,11,20-trione (21dE), and 5α-pregnan-17-ol-3,11,20-trione (11K-Pdione) were purchased from Steraloids (Wilton, USA). 5α-androstan-3α,11β,17β-triol (11OH-3αAdiol), 5α-androstan-11-one-3α,17β-diol (11K-3αAdiol), 5α-pregnan-11β-ol-3,20-dione (11βOH-DHP4), 5α-pregnan-3α,11β-diol-20-one (3,11diOH-DHP4), 5α-pregnan-11β,17α-diol-3,20-dione (11OH–Pdione), 5α-pregnan-3α,11β,17α-triol-20-one (11OH-Pdiol), 5α-pregnan-3α,17α-diol-11,20-dione (11K-Pdiol) and 5α-androstan-3-one-11β,17β-diol (11OHDHT) were purchased from IsoScience (Pennsylvania, USA). Cambridge Isotopes (Andover, USA) supplied the deuterated steroid reference standards; these include: deuterated progesterone 2,2,4,6,6,17A,21,21,21-D9, 98 % (D9-P4); deuterated 17α-hydroxyprogesterone 2,2,4,6,6,21,21,21-D8, 98 % (D8-17OHP4); deuterated 21-deoxycortisol 2,2,4,6,6,21,21,21-D8,_97 % (D8-21dF), and deuterated 11β-hydroxyandrostenedione 2,2,4,6,6,16,16-D7, 98 % (D7-11OHA4). Deuterated 11-ketodihydrotestosterone 16,16,17A-D3, ≥98 %_(D3-11KDHT) and deuterated 11-ketotestosterone 16,16,17A-D3, ≥98 % (D3-11KT) was purchased from Cayman Chemical company (Ann Arbor, Michigan, USA) and deuterated 11-keto-etiocholanolone 9A,12,12,16,16-D5, > 98 % (D5-11K-Etiocholanolone) purchased from CDN Isotopes (Augsburg, Bavaria, Germany). All other chemicals and reagents were purchased from reliable scientific supply houses.

2.2. Enzymatic assays in transiently transfected HEK-293 cells

HEK-293 cells used in the enzymatic assays were transiently transfected with expression vectors containing cDNA encoding human cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1). Cells were cultured using Corning® CellBIND® (100 mm plates and T75 flasks) in DMEM, pH 7.0, supplemented with 0.0015 % $NaHCO_3$ ($^m/_v$), 10 % FBS ($^v/_v$) and 1 % penicillin-streptomycin (10,000 U/mL and 10 mg/mL, respectively) at 37 °C, 90 % relative humidity and 5 % CO_2. After 3–5 passages, confluent cells were replated at 2×10^5 live cells/mL into Corning® CellBIND® surface 24-well plates. After 24 h, cells were transfected with 0.5 ng cDNA encoding CYP17A1. Transfected cells were incubated for 48 h after which steroid substrate was added in fresh DMEM and incubated for an addition *x* h pertaining to the particular time point. Media aliquots (500 μL) were collected and stored at 4 °C until extraction. The metabolism of steroid substrates in untransfected cells and HEK-293 cells transiently transfected with the pCI-neo plasmid (empty vector) were assayed in parallel with the enzymatic conversion assays as a negative control.

2.3. Steroid extraction

Steroids were extracted using a liquid-liquid extraction method and analysed using UPC^2-MS/MS. Prior to steroid extraction, media aliquots were spiked with internal standard consisting of deuterated steroid mix (1 ng D8-21dF, 1.5 ng D7-11OHA4, 10 ng D9-Prog and D8-17OHP4, 5 ng D3-11KDHT and D3-11KT, and 20 ng D5-11K-Etiocholanolone). MTBE was added at a ratio 3:1 (MTBE: media), and samples vortexed for 15 min at 1200 rpm using a MRC Multi-Tube Vortexer and placed at −80 °C. The organic phase was removed from the frozen aqueous phase, dried under nitrogen gas at 40 °C and the steroid residue re-suspended in 50 % HPLC-grade methanol in deionised water.

2.4. Steroid separation and quantification by UPC^2-MS/MS

Steroid standards, 2 mg/mL, were dissolved in absolute ethanol and used to prepare five steroid master mixes, 0.1, 10, 1000, 2000, 5000 ng/mL steroid, in 50 % methanol. The reference steroid standard range (0.001 − 2000 ng/mL) was prepared by spiking DMEM, 500 μL, with an appropriate volume of the steroid master mix containing the relevant steroids. Internal standards were added, and the samples extracted as described in Section 2.3. C11-oxy C_{19} and C11-oxy C_{21} steroids (Table 1) were separated on a AQUITY UPC^2 ethylene-bridged hybrid 2-ethylpyridine (BEH 2-

Table 1
Steroid standards used in the UPC^2-MS/MS analysis of the C11-oxy C_{19} and C11-oxy C_{21} steroids. Included were deuterated internal standards and quality control reference standards. Chemical names are depicted with their molecular weight (Mr), molecular ion species and fragment ion mass transitions. Method validation was specific to tissue culture medium as a matrix showing calibration range (ng/mL) and linearity (r^2) with the limit of detection (LOD) and limit of quantification (LOQ) shown as ng/mL and pg on column.

Compounds		Mr	Mass transitions			LOD*		LOQ*		Calibration range†	r²†
Abbreviation	IUPAC name		Quantifier	Qualifier	Qualifier	ng/mL	pg on column	ng/mL	pg on column		
Steroid metabolites											
11K-5αDione	5α-androstan-3,11,17-trione	302.41	303.2 > 241	303.2 > 227.1	303.2 > 248.9	1	6.67	2.5	16.67	2.5–1500	0.9984
11K-DHP4	5α-pregnan-3,11,20-trione	330.46	331.2 > 277.3	331.2 > 255	331.2 > 268.8	0.1	0.67	0.25	1.67	0.25–2000	0.9976
11KA4	4-androsten-3,11,17-trione	300.4	301.2 > 121.1	301.2 > 265.21	301.2 > 257	0.1	0.67	0.25	1.67	0.25–500	0.9957
11KP4	4-pregnen-3,11,20-trione	328.45	329.21 > 121	329.21 > 285.11	329.21 > 84.8	0.25	1.67	1	6.67	1.0–500	0.9992
11OH-5αDione	5α-androstan-11β-ol-3,17-dione	304.4	305 > 269.2	305 > 211	305 > 97.2	1	6.67	2.5	16.67	2.5–1000	0.9985
11βOH-DHP4	5α-pregnan-11β-ol-3,20-dione	332.48	333.1 > 85	333.1 > 159	333.1 > 279.25	< 10		10	66.67	10–1000	0.9988
11KAST	5α-androstan-3α -ol-11,17-dione	304.4	287 > 229.1	287 > 147.2	333.1 > 211.25	1	6.67	2.5	16.67	2.5–2000	0.9985
11K-Pdione	5α-pregnan-17-ol-3,11,20-trione	346.5	347.4 > 329	347.4 > 311.1	347.4 > 251	2.5	16.67	10	66.67	10–1250	0.9943
11OHA4	4-androstene-11β-ol-3,17-dione	302.4	303.2 > 267.2	303.2 > 285.2	303.2 > 121.1	0.25	1.67	1	6.67	1–1250	0.9997
11αOH-DHP4	5α-pregnan-11α-ol-3,20-dione	332.5	333 > 297.3	333 > 85.2	333 > 279.1	1	6.67	2.5	16.67	2.5–1250	0.9987
Alfaxalone	5α-pregnan-3α-ol-11,20-dione	332.48	333.2 > 214.96	333.2 > 297	333.2 > 85	1	6.67	2.5	16.67	2.5–500	0.9952
11KDHT	5α-androstan-17β-ol-3,11-dione	304.4	305.2 > 251	305.2 > 144.9	305.2 > 118.8	0.25	1.67	1	6.67	1–500	0.9984
11βOHP4	4-pregnen-11β-ol-3,20-dione	330.46	331.2 > 295.01	331.2 > 313	331.2 > 121.15	0.1	0.67	0.25	1.67	0.25–1250	0.9960
21dE	4-pregnen-17-ol-3,11,20-trione	344.44	345.2 > 163.2	345.2 > 121.08	345.2 > 105	0.1	0.67	0.25	1.67	0.25–1250	0.9977
11αOHP4	4-pregnen-11α-ol-3,20-dione	330.46	331.2 > 295.2	331.2 > 313.04	331.2 > 120.9	0.25	1.67	1	6.67	1–1250	0.9951
11KT	4-androsten-17β-ol-3,11-dione	302.41	303.2 > 147.1	303.2 > 285	303.2 > 107.2	1	6.67	2.5	16.67	2.5–1500	0.9955
11OHAST	5α-androstan-3α,11β-diol-17-one	306.44	289 > 271.3	289 > 253.2	289 > 213	< 25		25	166.67	25–1500	0.9927
11βOH-Pdione	5α-pregnan-11β,17α-diol-3,20-dione	348.48	349.2 > 114.99	349.2 > 183.25	349.2 > 71.03	2.5	16.67	10	66.67	10–1500	0.9960
3,11diOH-DHP4	5α-pregnan-3α,11β-diol-20-one	334.49	299.4 > 159	299.4 > 95	299.4 > 119.1	2.5	16.67	10	66.67	10–2000	0.9901
11OHDHT	5α-androstan-3-one-11β,17β-diol	306.44	307 > 271	307 > 213.2	307 > 253	1	6.67	2.5	16.67	2.5–2000	0.9950
21dF	4-pregnen-11β,17-diol-3,20-dione	346.46	347.1 > 147.27	347.1 > 95.39	347.1 > 210.9	1	6.67	2.5	16.67	2.5–1500	0.9995
11K-Pdiol	5α-pregnan-3α,17α-diol-11,20-dione	348.48	331.2 > 289.24	331.2 > 271.32	331.2 > 253.05	1	6.67	2.5	16.67	2.5–1500	0.9910
11K-3αAdiol	5α-androstan-11-one-3α,17β-diol	306.46	307.2 > 271	307.2 > 253	307.2 > 159.13	0.25	1.67	1	6.67	1–1500	0.9905
11OHT	4-androsten-11β,17β-diol-3-one	304.42	305.3 > 269	305.3 > 121	305.3 > 105.1	0.01	0.07	0.025	0.17	0.025–100	0.9942
11OH-Pdiol	5α-pregnan-3α,11β,17α-triol-20-one	350.49	296.9 > 279.1	296.9 > 145.1	296.9 > 105.12	25	166.67	100	666.67	100–2000	0.9940
11OH-3αAdiol	5α-androstan-3α,11β,17β-triol	308.46	273 > 107	273 > 147.2	273 > 161	10	66.67	25	166.67	25–2000	0.9902
Prednisone	1, 4-pregnadien-17, 21-diol-3, 11, 20-trione	358.43	359 > 147	359 > 171		–		–		–	–
Deuterated steroids	**Chemical name**										
D9-P4	progesterone-D9 (2,2,4,6,6,17A,21,21,21-D9)	323.5	324.2 > 107.15	324.2 > 134.95	324.2 > 194.24						
D8-17OHP4	17α-hydroxyprogesterone-D8 (2,2,4,6,6,21,21,21-D8)	338.5	340.1 > 322	340.1 > 168.2	340.1 > 147.2						
D5-11K-Etiocholanolone	11-keto-etiocholanolone-D5 (9A,12,12,16,16-D5)	309.4	291.3 > 230.2	291.3 > 273.24	291.3 > 92.97						
D7-11OHA4	11β-hydroxyandrostenedione-D7 (2,2,4,6,6,16,16-D7)	309.4	310.1 > 128.02	310.1 > 168.4	310.1 > 134.95						
D3-11KDHT	11-ketodihydrotestosterone-D3 (16,16,17A-D3)	307.4	308.2 > 290.12	308.2 > 131.05	308.2 > 197.15						
D3-11KT	11-ketotestosterone-D3 (16,16,17A-D3)	305.4	306.2 > 212.95	306.2 > 109.24	306.2 > 171						
D8-21dF	21-deoxycortisol-D8 (2,2,4,6,6,21,21,21-D8)	354.5	355.03 > 319.28	355.03 > 125.15	355.03 > 180.24						
Quality Control compounds											
trans-stilbene oxide	–	196.2	197.19 > 105.12	197.19 > 76.99	–						
sulfamethoxazole	–	253.3	254.06 > 108.06	254.06 > 92.17	–						
thymine	–	126.1	127.05 > 53.97	127.05 > 55.95	127.05 > 109.97						
sulfamethizole	–	270.3	271.032 > 92.17	271.032 > 107.99	–						

* The LOD and LOQ for each steroid were defined as the lowest concentration with a signal to noise ratio (S/N) of > 3 for the quantifier ion (LOD), and S/N > 10 for the quantifier ion and S/N > 3 for the qualifier (LOQ).

† The calibration curve was selected as the lowest quantifiable concentration to the highest concentration abiding to the linear range. Linearity was determined using masslynx software with the lowest r2 of 0.9901.

EP) column [3.0 × 100 mm, 1.7 μm particle size] and Van Guard pre-column [2.1 × 5 mm, 3.5 μm] and analysed using UPC^2-MS/MS (Waters Corporation, Milford, USA). Steroids were detected and analysed using a Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation, Milford, USA) in multiple reaction monitoring (MRM) mode, using positive electrospray ionisation (ESI+) mode. The steroids were separated as described in supplementary Table 1 and the steroid metabolites listed and characterised as shown in Table 1 and supplementary Table 2, with the molecular and fragment ions selected based on optimal resolution and crosstalk. The chromatographic separation is shown in Fig. 2. Waters UPC^2 Quality Control (QC) reference material containing trans-stilbene oxide, thymine, sulfamethoxazole and sulfamethizole was used to monitor chromatographic performance. The standard range was used to determine the limit of detection (LOD), limit of quantification (LOQ), calibration range (ng/mL), and linearity (r^2) for each steroid (Table 1).

2.5. Docking of 11OHP4, 11KP4, and 5α-reduced metabolites in human CYP17A1

The steroids 11OHP4, 11OH-DHP4, 11KP4, and 11K-DHP4 were docked into the X-ray structure of CYP17A1/A105L/progesterone after mutating L105 back to the native Ala at this position, based on structural investigations previously described [26]. Similarly, 3,11diOH-DHP4 and alfaxalone were docked into the X-ray structure of CYP17A1/A105L/pregnenolone after mutating L105 back to the native Ala. All docking procedures were performed using various modules of Schrödinger Maestro suite (Schrödinger, LLC, New York, NY, 2017).

The X-ray crystal structures of CYP17A1 with P4 (PDB 4NKX) and CYP17A1 with P5 (PDB 4NKW) were retrieved from RCSB PDB database and atoms of chain A including heme and the associated ligand were preserved for docking. In both structures, mutation back to the wild type Ala at position 105 was accomplished by using Mutate module of Schrodinger. The CYP17A1 polypeptide was optimized and prepared for docking using the Maestro Protein Preparation Wizard to assess bond order and missing hydrogens. This was followed by energy minimization (OPLS3 force field). Gaps in the protein structures were not filled because they were distant from the active site. Water molecules were removed from the protein. The heme iron was assigned a ferric oxidation state. The Maestro Receptor Grid Generation module was then used to define a 15 × 15 × 15 Å grid centred on the co-crystallized ligands.

Steroid structures were prepared by modification of the progesterone and pregnenolone ligands in the 4NKX and 4NKW structures, respectively, using the Maestro 3D Build module. The Maestro LigPrep module was then used to generate conformers of each compound subjected to energy minimization (OPLS3 force field).

The resulting compounds were docked into the prepared CYP17A1 structures using the Maestro Glide module with XP (extra precision) and flexible ligand sampling. Ligands were constrained to within the generated grid. Initial poses (5,000) were generated for each compound. On the basis of the pose score, the top 400 were selected and subjected to energy minimization (OPLS3 force field). Finally, the highest 10 poses per compound were ranked by their Glide score, an approximation of the receptor-ligand complex binding energy. The top pose was analysed as previously reported [27]. The total free energy of binding ΔG_{bind} (kcal/mol) was determined using prime molecular mechanics energies combined with the generalized Born and surface area continuum solvation [28]. Residues within 8 Å from the ligand were defined as flexible during free energy calculation.

2.6. Statistical analysis

All conversion assays are representative of three or more independent experiments performed in triplicate and expressed as mean ± SEM. Statistical relevance of the results was analysed by GraphPad Prism 6 (GraphPad Software, Inc., CA, USA) using an unpaired *t*-test. Statistical significance was measured as a probability (p) value ≤ 0.05 (*$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

3. Results

3.1. The conversion of C11-oxy C21 steroid metabolites by human CYP17A1

In the adrenal glucocorticoid- and androgen pathways, as well as in

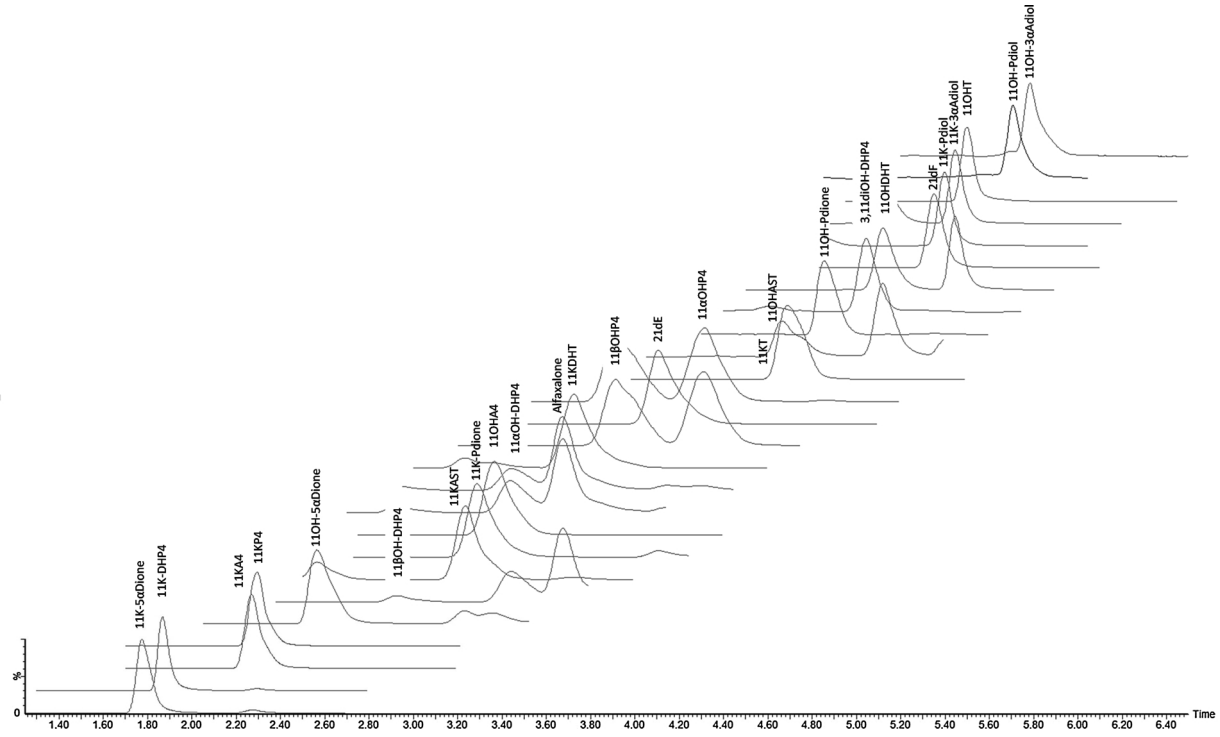

Fig. 2. Chromatogram of 26 C11-oxy C_{19} and C11-oxy C_{21} steroid reference standards generated by UPC^2-MS/MS.

the peripheral backdoor pathway, end-product formation is dependent on 17α-hydroxylase activity of CYP17A1 for cortisol production and subsequent 17,20-lyase activity to produce androgen precursors. In the backdoor pathway the 3α,5α-reduction of 17OHP4 to Pdiol—a C_{21} steroid, is a prerequisite for the CYP17A1-catalysed 17,20-lyase reaction yielding AST. We previously showed the conversion of 11OHP4 to 21dF [24], and that of 11KP4 and its reduced metabolites to C11,C17-products [25] by CYP17A1 but the involvement of the enzyme with regards to the C11-oxy C_{21} steroids in the C11-oxy backdoor pathway has not been fully explored. In this study we investigated the CYP17A1 activity in the top, middle and bottom tier steroids depicted in Fig. 1 of the C11-oxy backdoor pathway wherein the conversion of P4 and the backdoor Pdione and Pdiol pathway metabolites were included.

Comparable with previously published data [24,25], the 17α-hydroxylation of 1 μM 11OHP4 and 11KP4 yielded ~0.27 μM 21dF and ~0.65 μM 21dE after 12 h, respectively, with no 17,20-lyase activity detected. In addition, no lyase activity was detected towards 21dF or 21dE as substrates – the latter two already containing a hydroxyl group on C17 (Fig. 3A). After 12 h the metabolism of 1 μM 5α-reduced metabolites –11OH-DHP4, 11K-DHP4, 11OH-Pdione and 11K-Pdione (Fig. 3B) by CYP17A1 showed negligible conversion. The 17α-hydroxylation of 11OH-DHP4 to 11OH-Pdione and 11K-DHP4 to 11K-Pdione yielded 36.4 and 59.4 nM product, respectively. Negligible lyase activity was only detected towards 11OH-Pdione when assayed as substrate with the formation of low levels of 11OH-5αDione, whereas 11K-Pdione levels remained unchanged. The conversion of 1 μM 3α,5α-reduced metabolites (Figs. 3C; 4) by CYP17A1 showed a significant decrease in substrate concentrations. In the conversion of 3,11diOH-DHP4 ~90 % of the substrate was depleted after

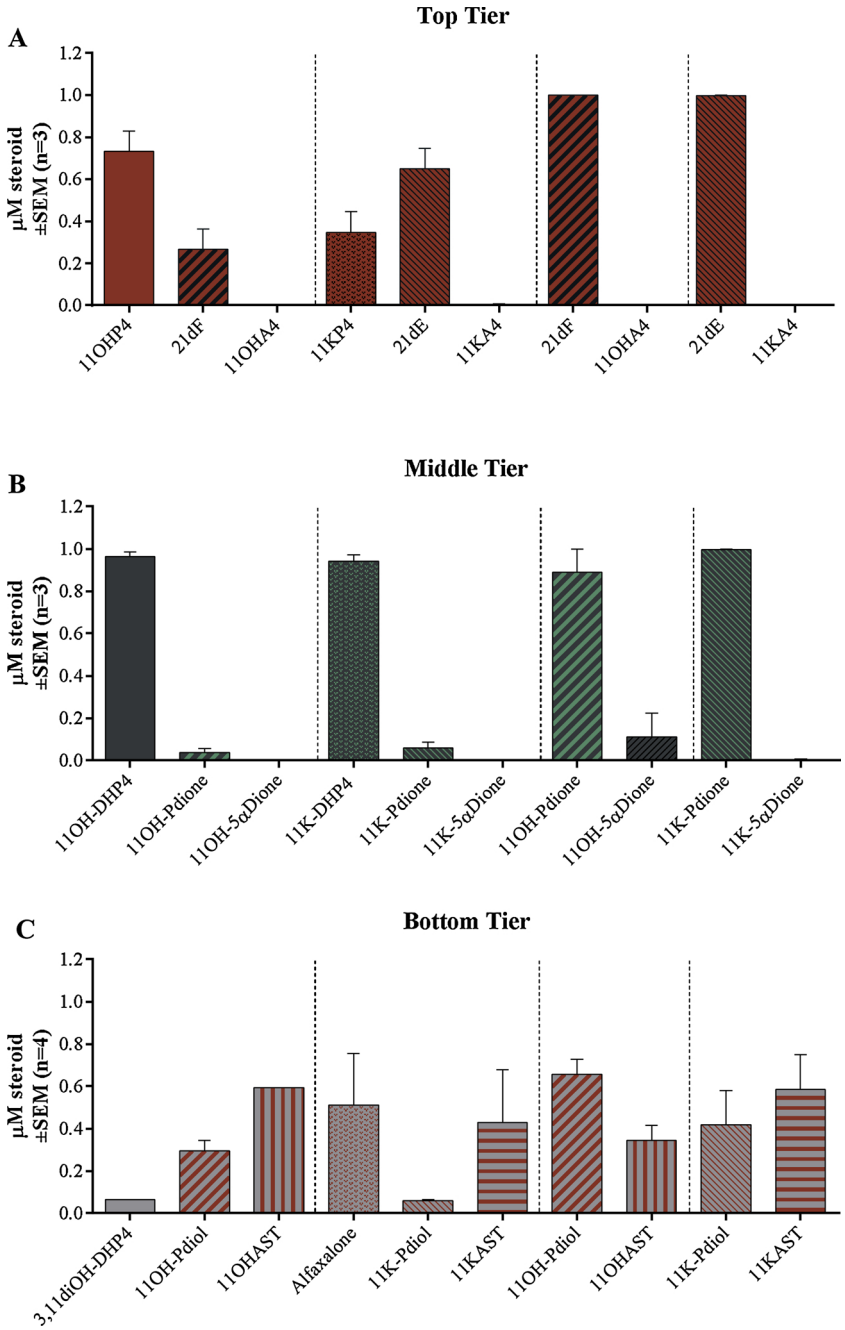

Fig. 3. Conversion of C11-oxy C_{21} steroids by CYP17A1. The 17α-hydroxylase/17,20-lyase activity towards (A) C11-oxy C_{21} steroids; (B) 5α-reduced C11-oxy C_{21} steroid metabolites and (C) 3α,5α-reduced C11-oxy C_{21} steroid metabolites after 12 h. Results are expressed as the mean ± SEM of at least three independent experiments performed in triplicate.

12 h (Fig. 3C), with the formation of 0.30 μM 11OH-Pdiol (17α-hydroxylated intermediate) and 0.59 μM 11OHAST (17,20-lyase end-product). A time course assay of the conversion of 3,11diOH-DHP4 by CYP17A1 over 24 h Fig. 4A showed full substrate conversion and the formation of 17αhydroxylated and 17,20-lyase products. After 9 h, comparable levels of 11OH-Pdiol (0.36 μM) and the lyase product, 11OHAST (0.32 μM) was detected; with 11OHAST concentrations then exceeding 11OH-Pdiol yielding 0.67 μM at 24 h. CYP17A1 also 17α-hydroxylated alfaxalone and the 11K-Pdiol intermediate was rapidly converted to 11KAST Figs. 3C; 4B). At 12 h, 51 % alfaxalone was metabolised to 59.8 nM 11K-Pdiol and 0.43 μM 11KAST. Although 11K-Pdiol concentrations remain constant over 24 h, 11KAST increased up to 0.92 μM at 18 h. Low levels of 11K-3αAdiol, 7.3 nM, were also detected which indicates conversion of 11KAST by endogenous 17β-hydroxysteroid dehydrogenases expressed in HEK-293 cells. Lastly, the CYP17A1 lyase activity assayed towards 11OH-Pdiol and 11K-Pdiol, the 17α-hydroxylated intermediate metabolites of 3,11diOH-DHP4 and alfaxalone, resulted in the production of C11-oxy C_{19} steroids. In the conversion of 11OH-Pdiol (Figs. 3C; 4C) 0.66 μM substrate was detected at 12 h after which 11OHAST production plateaued at ~0.35 μM. A slight fluctuation in 11OHAST and 11OH-Pdiol was observed towards 24 h. A higher conversion rate of 11K-Pdiol to 11KAST (Figs. 3C; 4D) compared to its C11 hydroxyl moiety was detected after 12 h with only 42 % substrate remaining. At 12 h the reaction peaked, producing 0.58 μM 11KAST, after which a similar fluctuating trend in 11OH-Pdiol conversion, was observed; 11K-Pdiol and 11KAST levels plateaued slowly, reaching an equilibrium at about 50 %.

Conversion of P4 showed high levels of 17OHP4 and with negligible levels of A4 as expected for the conversion by the human CYP17A1 (Fig. 5A). Similarly, DHP4—the 5α-reduced P4 metabolite, was readily hydroxylated to Pdione, with no lyase activity detected (Fig. 5B); whereas the C17-hydroxylated 3α,5α-reduced metabolite, Pdiol was rapidly converted to the C_{19} product (Fig. 5C).

3.2. Cortisol and its 3α,5α/β derivatives, 5α-tetrahydrocortisol and 5β-tetrahydrocortisol, are not substrates for the lyase activity of human CYP17A1 in vitro

11β-hydroxyandrostenedione (11OHA4) is a major adrenal androgen precursor biosynthesised by the CYP11B-catalysed 11β-hydroxylation of A4. Although we recently established the former reaction *in vitro*, earlier studies reported a minimal contribution to 11OHA4 pool by the side-chain cleavage of cortisol catalysed by an unknown enzyme. Axelrod et al. reported 1.5 and 1.4 % cortisol metabolised to 11OHA4 after 1 h incubation of human adrenal tissue with and without cofactors, respectively [29]. The same study reported ~2.4 % cortisol conversion to 11OHA4 in heparinised whole blood incubated with [1,2-^{3}H] cortisol and [4-^{14}C] A4, suggesting peripheral lyase activity towards cortisol. Urinary steroid profiles of healthy and diseased subjects showed 11OHAST, 11KAST and 11K-etiocholanolone as major metabolites, with numerous studies ascribing 11OHA4 as the precursor to the 5α-reduced C11-oxy C_{19} steroid and A-ring reduced cortisol as the precursor to the 5β-reduced androgens [30–35]. In addition, Shackleton et al. reported little difference between urinary C11-oxy C_{19} steroid levels (11OHAST, 11KAST and their 5β-reduced derivatives) in 17α-hydroxylase/17,20-lyase deficient (17OHD) patients treated with cortisol vs healthy controls [36]. Due to the lack of a functional CYP17A1 protein, the cleavage of the C17-20 bond of cortisol in 17OHD to C11-oxy androgens can only be facilitated by a desmolase other than CYP17A1. *In vivo* studies investigating the metabolism of radiolabelled 5α/5β-tetrahydrocortisol and 5β-tetrahydrocortisone detected urinary levels of 11OH- and 11K-etiocholanolone accounting for up to 22 % [34,37,38].

To clarify the catalytic activity of CYP17A1 towards cortisol and the A-ring reduced cortisol metabolites leading to the biosynthesis of C11-oxy C_{19} steroids, we assayed 1 μM cortisol, 5β-tetrahydrocortisol and 5α-tetrahydrocortisol in transiently transfected HEK-293 cells, ensuring high expression levels of human CYP17A1 limit potential interference of endogenous enzymes. Our data (Fig. 6) concur with previously published *in vitro* and *in vivo* studies showing no 11OHA4 formation from cortisol [36,39]. Similarly, neither the C17-20 bond of 5β-tetrahydrocortisol nor 5α-tetrahydrocortisol was cleaved by CYP17A1 after 24 h. No significant decrease in substrate concentrations was detected, providing further evidence that CYP17A1 does not exhibit lyase activity towards cortisol and its 3α, 5α/β-reduced metabolites *in vitro*.

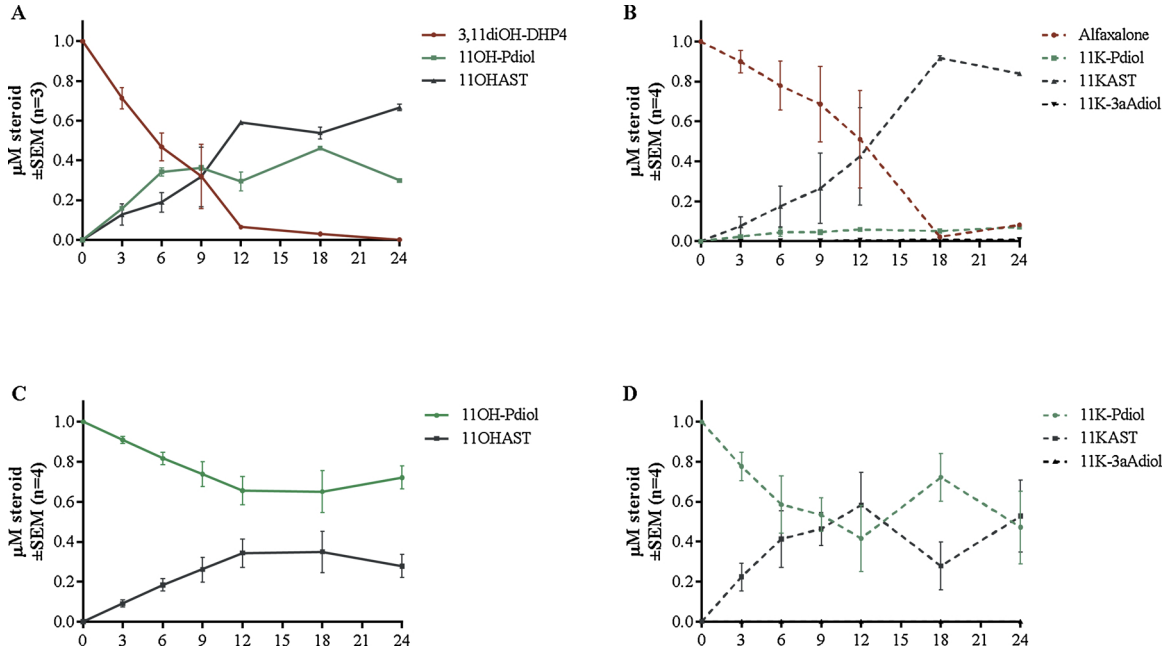

Fig. 4. CYP17A1 catalyses the conversion of 3α,5α-reduced C11-oxy C_{21} steroids to C11-oxy C_{19} steroids. The 17α-hydroxylase and 17,20 lyase reactions of (A) 3,11diOH-DHP4 and (B) alfaxalone yield 11OHAST and 11KAST. The 17,20 lyase reaction of the 17-hydroxylated intermediates (C) 11OH-Pdiol and (D) 11K-Pdiol yield 11OHAST and 11KAST. Endogenous 17β-hydroxysteroid dehydrogenases yields negligible 11K-3αAdiol. Results are expressed as the mean ± SEM of three or four independent experiments performed in triplicate n = 3–4).

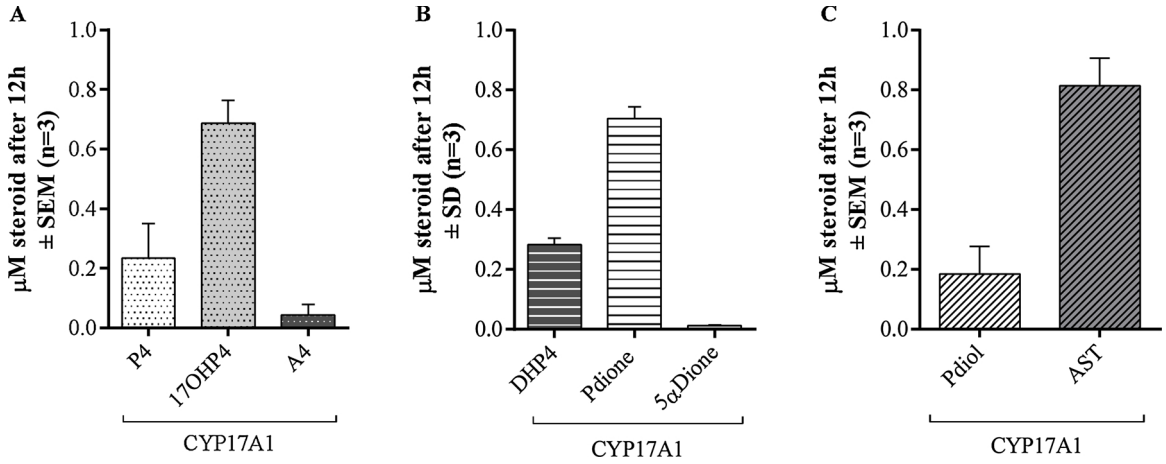

Fig. 5. Catalytic activity of CYP17A1 towards C_{21} steroids. The 17α-hydroxylase reaction of (A) P4 yields 17OHP4 and negligible A4; and (B) DHP4 yields Pdione and negligible 5αDione; while the 17,20 lyase reaction of Pdiol yields AST. Results are expressed as the mean ± SEM of three independent experiments performed in triplicate n = 3 while conversion of DHP4 is expressed as the mean ± SD of one experiment performed in triplicate n = 3.

3.3. Docking

11OHP4, 11KP4 and their 5α-reduced metabolites, 11OH-DHP4 and 11K-DHP4, were docked into the X-ray structure of CYP17A1/A105L/progesterone after mutating L105 back to the native Ala at this position. The docking steroidal cores overlapped very closely. The docked steroids had distances from C17 to the heme iron of 5.0 Å, 4.9 Å, 5.0 Å and 5.2 Å, respectively (Fig. 7A). Thus docking suggested that reduction of the Δ5 double bond does not seem to significantly modify the Fe-C17 distance. Each of these docking solutions have Fe-C17 distances similar to actual X-ray structures of CYP17A1 binding P4 itself (4.8 Å), which is consistent with little repositioning due to substitutions at C11. Docking also suggested that neither the C11 modifications nor reduction of Δ5 significantly modified the distance between the C3 keto group and N202 which were also highly conserved at 3.0–3.1 Å (Fig. 7A). This hydrogen bonding distance is very similar to that observed in X-ray structures for P4 (2.8 Å).

Similarly, 3,11diOH-DHP4 and alfaxalone were docked into the X-ray structure of CYP17A1/A105L/pregnenolone after mutating L105 back to the native Ala. The steroidal core of these two ligands also overlaid closely with each other, especially in the A and B rings, but the C and D rings of the 11β-OH molecule were positioned slightly farther from the I helix (Fig. 6B). As a result, the distances from C17 to the heme iron were 5.1 for the 11-keto compound *vs*. 5.7 Å for the 11β-OH compound. The alfaxalone C17-Fe distance was similar to those observed in X-ray structures with steroids with 3β-OH and 3-keto substrates (4.4 − 5 Å) and in the docking above, with the exception of 3,11diOH-DHP4. The 3α-hydroxyl is directed towards the F helix N202 side chain at a distance of 3.2–3.3 Å (Fig. 7B). This agrees with the distances from docking described above, as well as actual X-ray structures with steroids with variation of C3 substituents ranging from 3-keto, 3β-OH, [26] and 3α-OH (unpublished).

Overall the docking results here suggest a hydrogen bond between the N202 side chain and the C3 substituent, which is retained regardless of the A ring configuration and substitution at C11, and more variation in the C17-Fe distance. The C11 substituents all project into available space adjacent to the hydrophobic Val482 side chain and are too far from backbone atoms to form hydrogen bonds.

4. Discussion

CYP17A1 has been in the steroidogenic spotlight for decades due to its role regulating the direction of human steroidogenesis via its dual function, substrate specificity, tissue-specific expression and co-expression with other enzymes and cofactors. However, the enzyme's structural characteristics and specificity regarding the metabolism of C11-oxy C_{21} steroids via the backdoor pathway has remained unexplored. Our present study highlights the unique substrate specificity of CYP17A1, in terms of both the 17α-hydroxylase and the 17,20-lyase activity towards the C11-oxy C_{21} steroids, their 5α- and 3α,5α-reduced metabolites as demonstrated by the specific reactions catalysed by CYP17A1 and by the docking of 11OHP4, 11KP4, 11OH-DHP4 and 11K-DHP4 in the active site of the enzyme.

Exploring the catalytic activity of human CYP17A1, we showed the 17αhydroxylation of 11OHP4 and 11KP4 to 21dF and 21dE, respectively, with the hydroxylase reaction favouring the conversion of the C11-keto steroid while no products of the lyase reaction were detected (Fig. 3A). These reactions resemble the human CYP17A1 conversion of P4, a Δ^4-3-keto steroid to 17OHP4 with little to no biosynthesis of A4 (Fig. 5A) suggesting that the C11-oxy group does not cause a significant repositioning of 11OHP4 or 11KP4 in the active site. Structural studies investigating the catalytic activity of CYP17A1 towards the natural substrates, P5 and P4, reported two aspects influencing the hydroxylation reaction, further supporting our findings. Firstly, the presence of an Ala residue at position 105 in human and primate CYP17A1 result in an active site cavity increase which allows greater orientational freedom of P4, shifting both the C16 and C17 to the heme group for hydroxylation [26,40]. Although we did not assay the biosynthesis of the 16α-hydroxylated product due to the lack of a commercially available steroid standard, we did not observe a significant loss in

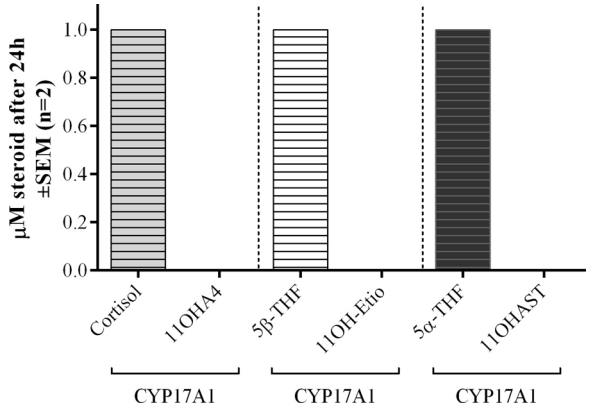

Fig. 6. Cortisol, 5β-tetrahydrocortisol (5β-THF) and 5α- tetrahydrocortisol (5α-THF) are not converted to C11-oxy C_{19} steroids by CYP17A1. Results are expressed as the mean ± SEM of two independent experiments performed in triplicate.

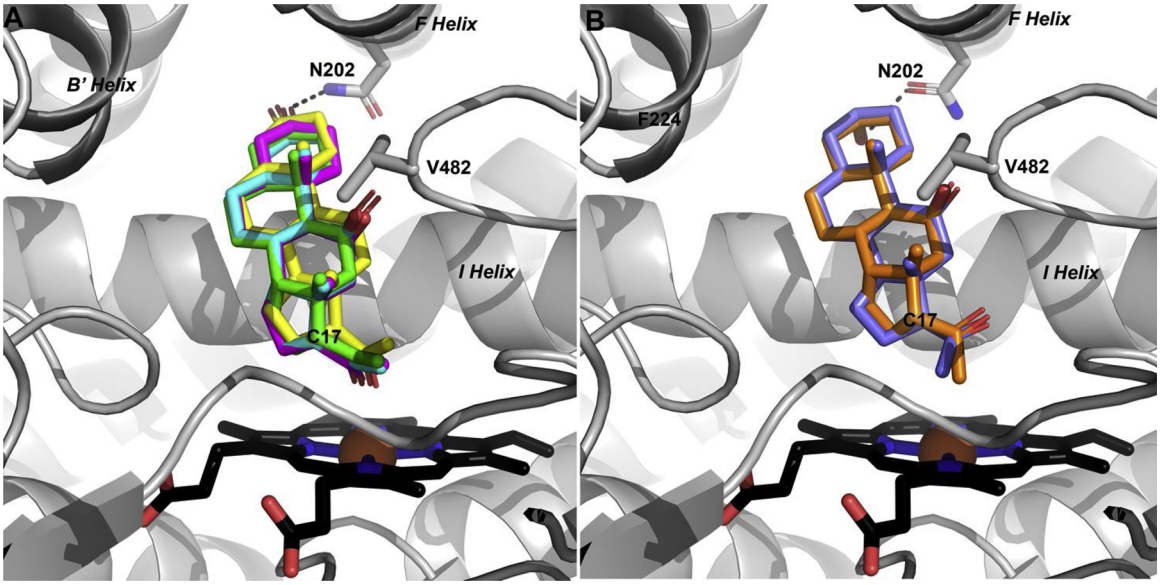

Fig. 7. Binding of C11-oxy C_{21} steroids in the active pocket of CYP17A1. (A) The steroids 11OHP4 (cyan), 11OH-DHP4 (magenta), 11KP4 (yellow) and 11K-DHP4 (green) were docked into CYP17A1. (B) The steroids 3,11diOH-DHP4 (orange) and alfaxalone (purple) were docked into CYP17A1. For clarity, only a single hydrogen bond is shown between N202 and the respective C3 substituents (black dashed line). The C17 atom is labelled.

substrate that was not accounted for by the 17α-hydroxylated product. However, further investigations are required to conclusively eliminate the potential formation of 11,16diOHP4. We previously reported the hydroxylation of 16OHP4 catalysed by the CYP11B isozymes [41], which may be of relevance in 21OHD characterised by abnormal levels of 16α-hydroxyprogesterone (16OHP4). The second aspect pertaining to the hydroxylase activity of CYP17A1 is the C3 moiety –its interaction with and distance to N202. In humans both 16OHP4 and 17OHP4 are produced from P4 catalysed by CYP17A1 whereas only 17OHP5 is biosynthesised from P5 [15]. The C3 hydroxyl group of P5 serves as a hydrogen bond acceptor or donor able to form stable and versatile interactions with the NH or oxygen group of N202 which could stabilize P5 in the active pocket occluding 16α-hydroxylation. In contrast, the C3-keto group of P4, can only act as a hydrogen bond acceptor and thus is limited to interaction with the NH group of N202. This could allow the A-ring of P4, but not P5, to shift closer to Ala105 and position C16 for hydroxylation. A slightly greater distance is observed between C3 and N202 for P5 (2.8 Å) compared to P4 (2.7 Å) while the C17-Fe distance for P5 and P4 are both 4.8 Å [26].

The 11-fold lower apparent K_m value reported for the hydroxylation of P5 (0.38 μM) compared to P4 (1.96 μM) and the significantly higher binding affinity of P5 > 17OHP5 > P4 > 17OHP4 (K_d < 100, 210, ~230 and 331 nM, respectively) [26,42] suggest that interactions of the steroid substrate in the active pocket facilitate the hydroxylation reaction. Taken together, and in the light of our structural findings of the close overlap of 11OHP4 (C17-Fe, 5.0 Å; C3-N202, 3.0–3.1 Å) and 11KP4 (C17-Fe, 4.9 Å; C3-N202, 3.0–3.1 Å) with P4 (C17-Fe, 4.8 Å; C3-N202, 2.8 Å) in the active site (Fig. 7A) these data support a similar orientation and positioning of 11OHP4 and 11KP4 with P4.

In the adrenal cortex, the zona-specific enzyme expression governs steroid biosynthesis. In the zona glomerulosa, the metabolism of P4 by cytochrome P450 21-hydroxylase (CYP21A2) and CYP11B2 promotes the biosynthesis of mineralocorticoids in the absence of CYP17A1. In contrast, the expression of CYP17A1 in the zona fasciculata (ZF) together with CYP21A2 and CYP11B1 regulates the production of glucocorticoids by hydroxylating P4 on C17 prior to being converted by CYP21A2 and CYP11B1. The ZF and zona reticularis (ZR) express high levels of CYP17A1, with cyt b_5 expressed in the latter augmenting the lyase reaction [43] to produce adrenal androgens. In 21OHD, a non-functional CYP21A2 results in deficient mineralocorticoid and glucocorticoid production, together with increased precursor metabolites, P4 and 17OHP4 [44]. Adrenal androgen production via the Δ^5-pathway is also increased as well as the C11-oxy C_{19} and C11-oxy C_{21} steroids [1,2]. The C11-oxy C_{19} steroids have been reported as the major adrenal androgen output in 21OHD together with decreased Δ^5-androgens (free and sulfated). In addition, immunostaining showed enzymatic integration between the ZF and ZR expressing CYP17A1, 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2) and cyt b_5 [2]. However, their findings showed that pregnenolone-sulfate concentrations were increased while the 17OHP5-sulfate levels were comparable to the control which suggests a shift to Δ^4-pathway or possibly other minor steroidogenic pathways. Although this study did not examine the C11-oxy C_{21} steroids, the enzymatic integration between the ZF and ZR, and availability of steroid precursors potentially enable Δ^4-3-keto-steroid production in both the ZF and ZR.

The biosynthesis of 11OHP4 and 21dF in the adrenal is furthermore supported by studies previously reporting the co-expression of CYP17A1 and CYP11B1 in the ZF and ZR [45,46] adding another layer of complexity with the potential 11β-hydroxylation of P4 and 17OHP4 in the three zonae, depending on the enzyme/substrate preference. It is possible for 11OHP4 to be hydroxylated at C17 and therefore contribute to 21dF levels, to which the high 21dF levels in 21OHD can be attributed. Our *in vitro* studies have shown that the preferential reaction for the production of 21dF is the 11β-hydroxylase of 17OHP4 instead of the 17α-hydroxylase of 11OHP4 [24].

The contribution of 11βHSD in the conversion of C11-oxy C_{21} steroids to their C_{19} derivatives cannot be discounted as we previously reported that the metabolism of 11OHP4 and 21dF in LNCaP cells transfected with the human CYP17A1 yielded C11-oxy C_{19} steroids via the keto derivatives [47] demonstrating the contribution of 11βHSD and CYP17A1 in the C11-oxy backdoor pathway. We have also shown that 11OHP4 is converted to 11KP4, 21dF and 21dE and subsequently to C11-oxy C_{19} steroids with the production of high levels of 11OHAST in the same transfected model [48]. Since the C4-C5 double bond reduction by SRD5A is an irreversible reaction, the detection of the 5α-reduced C11-oxy C_{19} steroids can only be due to the sequential 17α-hydroxylation and side-chain cleavage of 11KP4 metabolised by CYP17A1. Although CYP17A1 is not expressed in LNCaP cells, thus preventing the biosynthesis of C_{19} steroids from 11OHP4 [48], cyt b_5 is expressed (unpublished data) which, in the presence of the transfected CYP17A1, may account for the biosynthesis of 11OHA4 and its metabolites. These data generated in our *in vitro* model system in the

presence of steroidogenic enzymes, co-factors and co-enzymes, suggest that the metabolic pathways converting 11OHP4 and 11KP4 to C11-oxy C_{19} steroids are, in part, regulated by the co-enzymes with the metabolism by CYP17A1 in the absence of cyt b_5 possibly favouring the C11-oxy backdoor pathway. Alternatively, in the presence of cyt b_5, 11OHP4 and 11KP4 are converted to 11OHA4 and 11KA4. Further investigations are required to substantiate this since our data in the transiently transfected HEK-293 model system shows CYP17A1 to have no lyase activity towards 11OHP4, 11KP4, 21dF, or 21dE. In addition, while negligible 17α-hydroxylase activity was detected in the conversion of 11OH-DHP4 and 11K-DHP4 to 11OH-Pdione and 11KPdione, respectively, we also did not detect CYP17A1 lyase activity towards the 5α-reduced metabolites, 11OH-DHP4, 11K-DHP4, 11OH-Pdione, and 11KPdione. Although 11OHP4 and 11KP4 deviated functionally from the P4 metabolic pattern, post-5α-reduction our docking of 11OH-DHP4 and 11K-DHP4 into the active pocket showed minimal changes in the orientation compared to 11OHP4 and 11KP4, and P4. The compromised 17α-hydroxylase activity towards these steroids suggests functional interference by the C11-oxy group and/or 5α-reduction. Our report, supporting the finding by Gupta et al., showed that the 17α-hydroxylase conversion of DHP4, the 5α-reduced metabolite of P4, to Pdione was also followed by minimal formation of 5αDione, even after prolonged incubation periods [23]. Thus, DHP4 positioning in the active site is hindered once a C11-oxy group is added resulting in a minimal repositioning of the C11-oxy steroids relative to P4 which effects the hydroxylase activity of CYP17A1 significantly.

Structurally the 3α,5α-reduced steroids, 3,11diOH-DHP4, alfaxalone, 11OH-Pdiol and 11K-Pdiol resemble Pdiol, the backdoor pathway metabolite. Although we previously reported the conversion of alfaxalone to 11KAST demonstrating the lyase activity of CYP17A1 towards the 3α,5α-reduced steroid [25] we were unable to analyse the levels of the hydroxylated intermediate due to the steroid being unavailable commercially. In the current study we are now able to accurately quantify metabolites and show the rapid 17α-hydroxylation of both alfaxalone and 3,11diOH-DHP4 to 11K-Pdiol and 11OH-Pdiol and the subsequent lyase reaction yielding 11KAST and 11OHAST, respectively. The data shows that the conversion of 3,11diOH-DHP4 (~10 % remaining at 12 h) proceeded faster than that of alfaxalone (~50 % remaining after 12 h) −1 nmol/L/min *vs*. 0.566 nmol/L/min. These data suggest that the hydroxylase reaction of 3,11diOH-DHP4 is ~2 fold faster than that of alfaxalone with the rate of lyase reaction of the 11K-Pdiol intermediate similar to that of the 11OH-Pdiol intermediate (0.7 *vs*. 0.5 nmol/L/min) yielding higher levels of 11KAST than 11OHAST. Considering this data in context of *in vivo* analysis of hyperandrogenic diseases associated with elevated C11-oxy steroids in which 11OHAST and 11KAST have been considered biomarkers of 11OHA4-pathway activity, our data suggest that 3,11diOH-DHP4 and alfaxalone may contribute to the C11-oxy C_{19} pool with little indication or evidence of C11-oxy backdoor activity via the production of 11OH-Pdiol and 11K-Pdiol intermediates. Our findings reflect previous reports of the hydroxylase/lyase catalytic activity of CYP17A1 towards the 3α,5α-reduced P4, Pdiol. P4, once reduced to Pdiol, is rapidly converted to the C_{19} steroid, AST with negligible augmentation by cyt b_5 [23]. Interestingly, the conversion of 3,11diOH-DHP4 yielded higher levels of 11OHAST than the 17α-hydroxylated 11OH-Pdiol. Although this may be due to 11OH-Pdiol being more hydrophilic than 3,11diOH-DHP4, these data suggest a repositioning in the active site post-hydroxylation. Docking provided structural evidence of the close relation between 3,11diOH-DHP4 and alfaxalone in the human CYP17A1 active pocket. Interestingly, the shorter C17-Fe distance for 17OHP5 resulted in improved lyase activity when compared to that of 17OHP4 whereas the 3α,5α-reduced C11-oxy C_{21} steroids with a C17-Fe distance greater than 17OHP4 was rapidly cleaved yielding 11OHAST and 11KAST.

In conclusion, our data highlight the contribution of CYP17A1 in the metabolism of C11-oxy C_{21} steroids to C11-oxy C_{19} steroids and the potential role of the enzyme in downstream peripheral steroidogenic pathways. The expression of steroidogenic enzymes would, however, dictate pathways and outcomes. Although CYP17A1 will hydroxylate 11OHP4 and 11KP4 in the adrenal, the enzyme does not exhibit lyase activity towards their 17α-hydroxylated intermediates. The 17α-hydroxylation of the 5α-reduced C11-oxy C_{21} steroids, which would be produced in peripheral tissue expressing SRD5A, was negligible—it was only the 3α,5α-reduced C11-oxy C_{21} steroids which were readily hydroxylated, with the subsequent cleavage of the C17-20 bond yielding the C11-oxy C_{19} steroids, 11OHAST and 11KAST. Although the physiological relevance of the C11-oxy C_{21} steroids and their role in the endocrine system remains unexplored, these steroids would impact clinical conditions. The catalytic activity of CYP17A1 towards 11OHP4 would, for example, account for the production of 21dF in the 21OHD adrenal due to increased CYP21A2 precursor steroids. CYP17A1 transcripts have also been shown to be upregulated in abiraterone-resistant castration-resistant prostate cancer [49] which together with increased SRD5A and 3α-hydroxysteroid dehydrogenase expression [50] provide optimal conditions for C11-oxy C_{21} steroid metabolism in the backdoor pathway. Furthermore, the increase in hydrophilicity due to the reduction of the C11-oxy C_{21} steroids may also facilitate conjugation and elimination. However, we previously reported that the C11-oxy C_{19} steroids are poorly glucuronidated possibly due to the C11-oxy moiety [6] suggesting that these steroids may be reactivated to potent androgens while also remaining in circulation longer, contributing to the active androgen pool with adverse effects in target tissue. This may also be the case in the elimination of the C11-oxy C_{21} steroids as we recently identified these steroids in benign prostate hyperplasia, both in circulation and in tissue [8]. Taken together, our study places additional emphasis on the regulatory role of CYP17A1 in determining steroidogenic outcomes, not only in the adrenal but also in peripheral steroidogenic tissue.

Authors statement

Desmaré van Rooyen: *In vitro* enzyme assays, UPC^2-MS/MS method, original draft preparation; Rahul Yadav: Homology modelling; Emily E. Scott: Homology modelling, reviewing and editing; Amanda C. Swart: Conceptualization, supervision, writing and editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2020.105614.

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Supplementary table 1

Elution gradient specifications of the UPC^2 separation of C11-oxy C_{19} and C_{21} steroids

Step	Time (minutes)	Flow rate (mL/min)	Solvent A (%) (CO_2)	Solvent B (%) (methanol)	Gradient curve
1	initial	1.1	96	4	initial
2	1.3	1.1	92.6	7.4	5
3	1.5	1.1	92.5	7.5	6
4	3.2	1	91.4	8.6	6
5	3.6	0.9	87.4	12.6	6
6	4.0	0.85	87	13	6
7	4.6	0.825	86.4	13.6	6
8	4.8	1.1	75	25	6
9	5.0	1.1	75	25	6
10	5.1	1.1	96	4	6
11	6.5	1.1	96	4	6

Supplementary table 2

C11-oxy C_{19} and C_{21} steroid standards, deuterated internal standards and quality control reference standards included in the UPC^2-MS/MS method. Retention time (RT); molecular ion species and MRM mass transitions; mass spectrometer settings: cone voltages (CV), collision energy (CE).

Compounds	RT (min)	Mass transitions			CV (V)			CE (eV)		
		Quantifier	Qualifier	Qualifier						
Steroid metabolites										
11K-5αDione	1.70	303.2 > 241	303.2 > 227.1	303.2 > 248.9	35	35	35	20	20	20
11K-DHP4	1.80	331.2 > 277.3	331.2 > 255	331.2 > 268.8	25	25	25	15	20	20
11KA4	2.17	301.2 > 121.1	301.2 > 265.21	301.2 > 257	35	35	35	30	15	25
11KP4	2.20	329.21 > 121	329.21 > 285.11	329.21 > 84.8	15	15	15	20	20	20
11OH-5αDione	2.51	305 > 269.2	305 > 211	305 > 97.2	35	35	35	15	20	35
11βOH-DHP4	2.84	333.1 > 85	333.1 > 159	333.1 > 279.25	30	30	30	20	20	15
11KAST	3.09	287 > 229.1	287 > 147.2	333.1 > 211.25	30	30	30	20	30	20
11K-Pdione	3.22	347.4 > 329	347.4 > 311.1	347.4 > 251	25	25	25	15	15	20
11OHA4	3.26	303.2 > 267.2	303.2 > 285.2	303.2 > 121.1	30	30	30	15	15	25
11αOH-DHP4	3.26	333 > 297.3	333 > 85.2	333 > 279.1	30	30	30	15	20	15
Alfaxalone	3.48	333.2 > 214.96	333.2 > 297	333.2 > 85	25	25	25	15	15	25
11KDHT	3.56	305.2 > 251	305.2 > 144.9	305.2 > 118.8	30	30	30	15	30	30
11βOHP4	3.75	331.2 > 295.01	331.2 > 313	331.2 > 121.15	30	30	30	15	15	25
21dE	4.00	345.2 > 163.2	345.2 > 121.08	345.2 > 105	25	25	25	25	30	35
11αOHP4	4.04	331.2 > 295.2	331.2 > 313.04	331.2 > 120.9	30	30	30	15	15	25
11KT	4.51	303.2 > 147.1	303.2 > 285	303.2 > 107.2	30	30	30	25	15	30
11OHAST	4.56	289 > 271.3	289 > 253.2	289 > 213	15	15	15	15	15	15
11βOH-Pdione	4.79	349.2 > 114.99	349.2 > 183.25	349.2 > 71.03	25	25	25	30	30	20
3,11diOH-DHP4	4.96	299.4 > 159	299.4 > 95	299.4 > 119.1	30	30	30	25	30	30
11OHDHT	5.02	307 > 271	307 > 213.2	307 > 253	25	25	25	15	20	20
21dF	5.29	347.1 > 147.27	347.1 > 95.39	347.1 >210.9	25	25	25	20	40	20

11K-Pdiol	5.34	331.2 > 289.24	331.2 > 271.32	331.2 > 253.05	30	30	30	15	15	30
11K-3αAdiol	5.37	307.2 > 271	307.2 > 253	307.2 > 159.13	20	20	20	15	20	20
11OHT	5.45	305.3 > 269	305.3 > 121	305.3 > 105.1	35	35	35	15	20	20
11OH-Pdiol	5.66	296.9 > 279.1	296.9 > 145.1	296.9 > 105.12	25	25	25	15	30	40
11OH-3αAdiol	5.69	273 > 107	273 > 147.2	273 > 161	25	25	25	20	20	20
Prednisone	5.48	359 > 147	359 > 171		25	25	-	35	35	-
Deuterated steroids										
D9-Prog	1.55	324.2 > 107.15	324.2 > 134.95	324.2 >194.24	30	30	30	30	30	25
D8-17OHP4	2.92	340.1 > 322	340.1 > 168.2	340.1 > 147.2	26	26	26	15	15	25
D5-11K-Etiocholanolone	3.12	291.3 > 230.2	291.3 > 273.24	291.3 > 92.97	25	25	25	20	15	30
D7-11OHA4	3.23	310.1 > 128.02	310.1 > 168.4	310.1 > 134.95	25	25	25	25	30	30
D3-11KDHT	3.55	308.2 > 290.12	308.2 > 131.05	308.2 > 197.15	25	25	25	15	30	20
D3-11KT	4.53	306.2 > 212.95	306.2 > 109.24	306.2 > 171	15	15	15	20	30	30
D8-21dF	5.27	355.03 > 319.28	355.03 > 125.15	355.03 > 180.24	20	20	20	15	25	20
Quality Control compounds										
trans-stilbene oxide	0.64	197.19 > 105.12	197.19 > 76.99	-	25	25	-	20	30	-
sulfamethoxazole	5.81	254.06 > 108.06	254.06 > 92.17	-	30	30	-	25	30	-
thymine	3.10	127.05 > 53.97	127.05 > 55.95	127.05 > 109.97	25	25	25	20	20	20
sulfamethizole	Not eluted	271.032 > 92.17	271.032 > 107.99	-	30	30	-	30	20	-

Declaration by the candidate:

With regard to Chapter 4, pages 73-87, the nature and scope of my contribution were as follows:

Nature of contribution: Desmaré van Rooyen completed the following: generated the bulk of the data reported in the article and all the data in the supplemental file; developed the UPC^2-MS/MS method used for steroid analysis, analysed and processed all the data; complied all tables and figures with the exception of Figure 7, the graphical abstract and the steroid schematic (Figure 1); compiled the first draft of the manuscript, researched articles relevant to manuscript used in the introduction and discussion.

Extent of contribution: total generated data in manuscript and supplemental file, 90%; manuscript first draft 90%; joint editing of manuscript (50%) with AC Swart.

The following co-authors have contributed to Chapter 4, pages 73-87:

Name; e-mail address; nature of contribution; extent of contribution (%)

1. Rahul Yadav: ryadav@med.umich.edu; generated data together with Emily E. Scott pertaining to the docking shown in Figure 7, contributing 20 % of data reported in the manuscript. Read the manuscript draft.
2. Emily E. Scott: scottee@med.umich.edu; compiled the first draft with regards to the result section of docking study. Read and edited the final manuscript draft.
3. Amanda C. Swart; acswart@sun.ac.za; principal investigator and corresponding senior author.

Signature of candidate: *_____

Date: 10 January 2020

Declaration by co-authors:

The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapter 4, pages 73-87
2. no other authors contributed to Chapter 4, pages 73-87 besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapter 4, pages 73-87 of this dissertation.

Signature	Institutional affiliation	Date
*_____	University of Michigan & Mississippi State University	7 January 2020
*_____	University of Michigan	7 January 2020
*_____	Stellenbosch University	10 January 2020

*Declaration with signature in possession of candidate and supervisor.

Chapter 5

11β-hydroxysteroid dehydrogenase isoforms catalyse the interconversion of C11-oxy C_{21} steroids in the C11-oxy backdoor pathway

5.1 Introduction

Over the years, research regarding 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) and type 2 (11βHSD2) have been largely related to the activation and inactivation of glucocorticoids, with the focus being on cortisol and cortisone. Cortisol binds both the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) and due to its high affinity for the MR and high circulating levels competes with aldosterone, the natural MR ligand (Tannin *et al.* 1991, White *et al.* 1997, Chapman *et al.* 2013). The 11βHSD2-catalysed oxidation of cortisol to inactive cortisone ensures the regulation of glucocorticoid action whereas 11βHSD1 in the presence of hexose-6-phosphate dehydrogenase (H6PDH) catalyse the reverse reaction and the re-activation of cortisol. In addition, the 11βHSD isozymes catalyse the C11-oxy C_{19} steroids in the 11OHA4 pathway. Adrenal 11β-hydroxyandrostenedione (11OHA4) and 11β-hydroxytestosterone (11OHT) are converted to 11-ketoandrostenedione (11KA4) and 11-ketotestosterone (11KT) by 11βHSD2 and are subsequently metabolised to yield 11-ketodihydrotestosterone (11KDHT). Although 11OHT shows androgenic activity towards the androgen receptor (AR), 11KT and 11KDHT are reportedly more androgenic, with androgenicity similar to testosterone (T) and dihydrotestosterone (DHT) (Swart *et al.* 2013, Bloem *et al.* 2015). Thus, the complex role of the 11βHSD isozymes is emphasised in the inactivation of glucocorticoids by 11βHSD2 catalysing the oxidation reaction whereas, in contrast, the androgenic activity of C11-oxy C_{19} steroids is increased by the same reaction.

Considering the aforementioned and the increase in reports regarding elevated C11-oxy C_{21} steroids in clinical conditions, these enzymes have, yet again, been placed under the spotlight with the focus directed towards C11-oxy C_{21} steroid metabolism. We recently reported on the metabolism of 11β-hydroxyprogesterone (11βOHP4), 11-ketoprogesterone (11KP4), 21-deoxycortisol (21dF) and 21-deoxycortisone (21dE) by the steroidogenic enzymes of the backdoor pathway (Barnard *et al.* 2017, Van Rooyen *et al.* 2018, Gent, du Toit, Bloem, *et al.* 2019) as well as conversion the 5α,3α-reduced C11-oxy C_{21} metabolites to C11-oxy C_{19} steroids

catalysed by cytochrome P450 17α-hydroxylase /17,20-lyase (CYP17A1) (Van Rooyen *et al.* 2020). Although 11βOHP4 reportedly exhibits inhibitory properties towards 11βHSD2 (Gent, du Toit, Bloem, *et al.* 2019), 11βOHP4 and 11KP4 are substrates for 11βHSD2 and 11βHSD1, respectively. However, the catalytic activity of the 11βHSD isozymes towards the C11-hydroxy and C11-keto C_{21} backdoor pathway metabolites remain unexplored. In this chapter, we aimed to elucidate the extent of the involvement of 11βHSD in the C11-oxy backdoor pathway by assaying the conversion of 11βOHP4, 21dF and their 5α- and 5α,3α-metabolites by 11βHSD2 and the conversion of their keto-derivatives by 11βHSD1 co-expressed with H6PDH in HEK-293 cells. In addition, we investigated the oxidation of 5α-androstan-3α,11β,17β-triol (11OH-3αAdiol) and 5α-androstan-3α,11β-diol-17-one (11OHAST) by 11βHSD2 and reduction of 5α-androstan-11-one-3α,17β-diol (11K-3αAdiol) and 5α-androstan-3α-ol-11,17-dione (11KAST) by 11βHSD1. Metabolism of 11βOHP4 and 21dF in the androgen-independent castration resistance prostate cancer cell line (C4-2B), which expresses 11βHSD2 endogenously (Du Toit and Swart 2018), was also investigated.

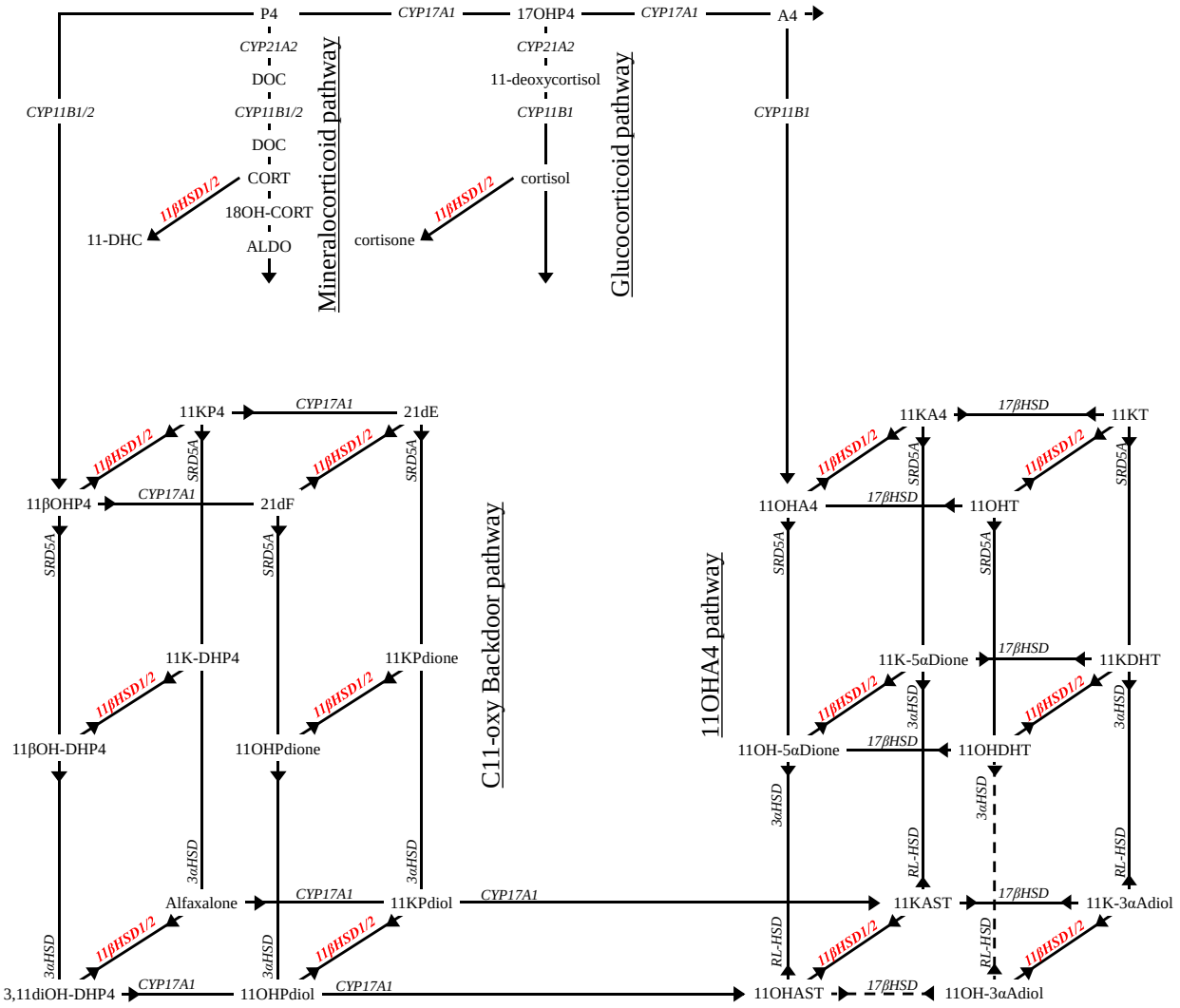

Figure 5. 1 11β-hydroxysteroid dehydrogenase type 1 and type 2 catalyse the peripheral conversion of C11-oxy steroids in the mineralocorticoid, glucocorticoid, C11-oxy backdoor and 11OHA4 pathways.

5.2 Materials and methods

5.2.1 Materials

Methyl *tert*-butyl ether (MTBE), analytical-grade methanol, formic acid, Dulbecco's Modified Eagle's Medium (DMEM), D-(+)-Glucose, Roswell Park Memorial Institute 1640 (RPMI-1640) medium and steroids including 5-pregnen-3β-ol-20-one (P5), 5-pregnen-3β,17α-diol-20-one (17OHP5), 5-androsten-3β-ol-17-one (DHEA), 4-pregnen-3,20-dione (P4), 4-pregnen-17α-ol-

3,20-dione (17OHP4), 4-pregnen-16α-ol-3,20-dione (16OHP4), 4-androsten-3,17-dione (A4), 4-androsten-17β-ol-3-one (T), 5α-androstan-17β-ol-3-one (DHT), dihydroprogesterone (DHP4), 4-pregnen-21-ol,3,20-dione (DOC), 4-pregnen-11β,21-diol-3,20-dione (CORT), 4-pregnen-11β,21-diol-3,18,20-trione (ALDO), 4-pregnen-17α,21-diol-3,20-dione (11-deoxycortisol), 4-pregnen-17,21-diol-3,11,20-trione (cortisone), 4-pregnene-11β, 17α,21-triol-3,20-dione (cortisol), 4-pregnen-11β,18,21-triol-3,20-dione (18OH-CORT) and 1,4-pregnadien-17α,21-diol-3,11,20-trione (prednisone) were purchased from Sigma-Aldrich (St. Louis, USA). The human prostate cancer C4-2B LNCaP-derived cell model was gifted by Prof. D.E. Neal (Nuffield Department of Surgery, Oxford, UK) and HEK-293 cells were purchased from the American Type Culture Collection, ATCC (Manassas, USA). Sodium pyruvate and HEPES were purchased from Biochrom (Berlin, Germany). Fetal bovine serum (FBS), trypsin-EDTA and penicillin-streptomycin (Pen-Strep) were supplied by ThermoFisher Scientific. Corning® CellBIND® surface tissue cultureware (T75 flasks, 100mm dishes, and 24-well plates) were purchased from Corning® Life Science (NY, USA), and trypan blue (0.4%) stain and Countess® cell count plates from Invitrogen (Eugene, USA). X-tremeGENE HP® DNA transfection reagent was obtained from Roche Diagnostics (Mannheim, Germany). 4-pregnen-11β-ol-3,20-dione (11βOHP4), 4-pregnen-3,11,20-trione (11KP4), 5α-pregnan-3,11,20-trione (11K-DHP4), 5α-pregnan-3α-ol-11,20-dione (alfaxalone), 4-pregnen-11β,17-diol-3,20-dione (21dF), 4-pregnen-17-ol-3,11,20-trione (21dE), 5α-pregnan-17α-ol-3,11,20-trione (11K-Pdione), 4-androstene-11β-ol-3,17-dione (11OHA4), 4-androsten-3,11,17-trione (11KA4), 5α-androstan-3α,11β-diol-17-one (11OHAST) and 5α-androstan-3α -ol-11,17-dione (11KAST) were purchased from Steraloids (Newport, USA). 5α-pregnan-11β-ol-3,20-dione (11βOH-DHP4), 5α-pregnan-3α,11β-diol-20-one (3,11diOH-DHP4), 5α-pregnan-11β,17α-diol-3,20-dione (11OH-Pdione), 5α-pregnan-3α,11β,17α-triol-20-one (11OH-Pdiol), 5α-pregnan-3α,17α-diol-11,20-dione (11K-Pdiol), 5α-androstan-3α,11β,17β-triol (11OH-3αAdiol), 5α-androstan-11-one-3α,17β-diol (11K-3αAdiol) and 5α-androstan-11β,17β-diol-3-one (11OHDHT) were purchased from IsoScience (Pennsylvania, USA). Deuterated progesterone 2,2,4,6,6,17A,21,21,21-D9, 98% (D9-P4); deuterated 17α-hydroxyprogesterone 2,2,4,6,6,21,21,21-D8, 98% (D8-17OHP4); deuterated 21-deoxycortisol 2,2,4,6,6,21,21,21-D8, 97 % (D8-21dF), and deuterated 11β-hydroxyandrostenedione 2,2,4,6,6,16,16-D7, 98% (D7-11OHA4) were supplied by Cambridge Isotopes (Andover, USA). Deuterated 11-ketodihydrotestosterone 16,16,17A-D3, ≥98 % (D3-

11KDHT) and deuterated 11-ketotestosterone 16,16,17A-D3, ≥98 % (D3-11KT) were purchased from Cayman Chemical company (Ann Arbor, Michigan, USA) and deuterated 11-keto-etiocholanolone 9A,12,12,16,16-D5, >98% (D5-11K-Etiocholanolone) was purchased from CDN Isotopes (Augsburg, Bavaria, Germany). All other chemicals were sourced from reliable scientific supply houses.

5.2.2 Plasmid constructs and plasmid preparation for transiently transfected enzymatic assays

The human 11βHSD1/pCR3, 11βHSD2/pCR3 and H6PDH/pcDNA3.2 were purchased from Prof P. Stewart (University of Leeds, UK) and pcDNA3.1/hCYP17A1 from Prof. A.V. Pandey (University of Bern, Switzerland). pCIneo and the Promega Wizard® Plus Midipreps DNA purification system were purchased from Promega (Madison, USA). *Escherichia coli* cells (JM109 strain) transformed with the plasmid cDNA of interest were inoculated in Luria-Bertani (LB) medium containing ampicillin (100 µg/mL), incubated for ± 15 hrs at 37 °C on an Innova shaking incubator (New Brunswick) at 220 rpm. The plasmid DNA was purified in accordance with the manufacturer's instructions and stored at -20 °C. Prior to transfection, the plasmid constructs were validated by restriction enzyme digest followed by agarose gel electrophoresis and subsequently sequenced. Plasmid concentrations were determined by UV absorption at 260 and 280 nm using a Cary 60 UV-Vis (Agilent technologies, California, USA).

5.2.3 Enzymatic assays in transiently transfected HEK-293 cells

The conversions catalysed by 11βHSD isozymes were investigated in human embryonic kidney cells (HEK-293) co-transfected with (i) the cDNA expression vector encoding 11βHSD2 and pCIneo (without insert cDNA), and (ii) the cDNA expression vector encoding 11βHSD1 and the cDNA expression vector encoding glucose-6-phosphate dehydrogenase (H6PDH). HEK-293 cells were cultured using Corning® CellBIND® tissue cultureware in DMEM, pH 7.0, supplemented with $NaHCO_3$ (0.15% $^{m}/_{v}$), FBS (10% $^{v}/_{v}$) and 1% penicillin-streptomycin (Pen-Strep) (10,000 U/mL and 10 mg/mL, respectively) at a temperature, relative humidity and CO_2 levels maintained at 37 °C, 90% and 5%, respectively. Confluent cells between the passage of 3-5 were replated at 2 x 10^5 live cells/mL into 24-well plates and incubated for 24 h prior to transfection using a total of 0.5 µg cDNA encoding 11βHSD1 and H6PDH (1:1), or 11βHSD2 and pCINeo (1:1). After 48 h incubation, steroid substrate, 1 µM, was added in fresh DMEM and incubated for an additional

24 h after which 500 µL media aliquots were collected and stored at 4 °C until extraction. Negative controls were included in parallel in all assays in which the conversion of the corresponding substrate was assayed in untransfected cells and in cells transiently transfected with pCIneo (without insert cDNA).

5.2.4 Steroid assays in C4-2B cells

The androgen-independent C4-2B cells were cultured in RPMI-1640 medium, pH 7.0, supplemented with the following: 1.5g $NaHCO_3$/L, 2.5 g D-(+)-Glucose/L, 10% FBS ($^{v}/_{v}$), 1% Pen-Strep (10,000 U/mL and 10 mg/mL, respectively), 1 mM sodium pyruvate and 10 mM HEPES. Cells were cultured in Corning® CellBIND® tissue culture ware and incubated at 37 °C, 90% relative humidity and 5 % CO_2. After 3-5 passages, cells were replated at 2 x 10^5 live cells/mL into T75 flasks and incubated for 24 hrs after which the cells were transiently transfected with 20 µg plasmid cDNA encoding human CYP17A1. After 24 hrs, transfected cells were replated at 2 x 10^5 live cells/mL into 12-well plates and incubated for another 24 hrs followed by steroid substrate addition, 1000 µL, prepared in fresh RPMI medium. After 96 hrs, media aliquots, 500 µL, were removed and stored at 4 °C until extraction. Controls were assayed in parallel with the experiment and included the following: metabolism of substrate in C4-2B cells transiently transfected with pCIneo (vector without insert); metabolism of 11OHA4 in transfected C4-2B cells as a positive control and compared to published results (Du Toit and Swart 2018); metabolism of Pdiol in transfected C4-2B as well as HEK-293 cells cultures in parallel to assess the transfection efficiency. 500 µL aliquots of media containing the steroid substrate were taken at the time of substrate addition (T_0) and analysed to validate substrate concentrations.

5.2.5 UPC^2-MS/MS steroid analysis and quantification

Steroids were extracted from media using a MTBE liquid-liquid extraction protocol and analysed using the UPC^2-MS/MS method previously described in chapter 4. Briefly, media aliquots were spiked with an internal standard mix prior to the addition of 1.5 mL MTBE. Samples were vortexed, frozen at -80 °C and the organic phase removed and dried under nitrogen gas at 40 °C. Steroid residue was resuspended in analytical-grade methanol (50%, prepared in deionised water) and stored at -20 °C until analysis.

Steroids were separated on a AQUITY UPC^2 ethylene-bridged hybrid 2-ethylpyridine (BEH 2-EP) column [3.0 x 100 mm, 1.7 µm particle size] connected with a Van Guard pre-column [2.1 x 5 mm, 3.5 µm] and analysed in multiple reaction monitoring (MRM) mode, using positive electrospray ionisation (ESI+) mode using a Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation, Milford, USA). Steroids were characterised and separated as previously described in chapter 4.

5.2.6 Statistical analysis

Statistical analysis was carried out with GraphPad Prism 6 (GraphPad Software, Inc., CA, USA) using an unpaired *t*-test. Statistical significance was measured as a probability (p) value ≤ 0.05 (*$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Results are, unless specified otherwise, representative of three or more independent experiments performed in triplicate and expressed as mean ±SEM.

5.3 Results

5.3.1 The oxidation and reduction of C11-oxy C_{21} steroids by the 11βHSD isoforms

While the 11βHSD isoforms have been reported to interconvert both glucocorticoids and C11-oxy androgens as well as the C11-oxy progesterones (11βOHP4, 11KP4, 21dF and 21dE), the oxidation and reduction by 11βHSD isoforms of these downstream C11-oxy C_{21} steroid metabolites remained unresolved due to the unavailability of reference standards. We therefore investigated the 11βHSD-catalysed interconversion of C11-hydroxyl and C11-keto C_{21} steroids and their metabolites in HEK-293 cells co-transfected with either 11βHSD1 and H6PDH or with 11βHSD2 and pCIneo. Comparing the catalytic activities of 11βHSD1 and 11βHSD2 , a multiple t-test analysis of the percentage product formation showed no statistical difference in their activity towards their respective substrates, with the exception of 11OH-Pdiol and 11K-Pdiol –evident in the conversion of 1 µM C11-hydroxyl C_{21} steroid metabolites (solid bars) by 11βHSD2 (Figure 5.2; top) to their C11-keto moieties and the conversion of the C11-keto C_{21} steroid metabolites (banded bars) by 11βHSD1 (Figure 5.2; bottom) to their C11-hydroxyl moieties. After 24 h, the conversion of 11βOHP4 yielded ~0.80 µM 11KP4 whereas 11βOH-DHP4 and 3,11diOH-DHP4 showed a negligibly lower product formation of ~0.69 and ~0.65 µM 11K-DHP4 and alfaxalone, respectively.

In the conversion of 11,17 dihydroxy metabolites —21dF, 11OHPdione and 11OHPdiol, a negligibly higher conversion was observed compared to the C11-oxy steroids which do not have the C17 hydroxyl moiety, with only ~0.27, ~0.15 and ~0.18 µM substrate remaining after 24 h. The 11βHSD1 investigations emphasised the interchange between 11βOHP4 and 11KP4 being efficiently catalysed by both enzymes with 85% 11KP4 converted to 11βOHP4. However, an opposite trend is observed for 21dE and the 3α.5α-reduced C11-oxy C_{21} metabolites with high levels of the substrate (keto derivatives) remaining after 24 h. The 21dE and the 5α-reduced metabolites, 11K-DHP4 and 11K-Pdione, showed ~25-34% product formation whereas the conversion of alfaxalone and 11K-Pdiol (3α,5α-reduced keto metabolites) yielded only ~16 and ~22% 3,11diOH-DHP4 and 11OH-Pdiol, respectively. As a positive control, the conversion of 11OHA4 by 11βHSD2 and 11KA4 by 11βHSD1 were assayed in parallel and corresponded with previously published results (Swart *et al.* 2013). In addition, substrates were assayed in untransfected cells and HEK-293 cells transiently transfected with pCIneo to monitor endogenous conversions. Endogenous reductase activity has previously been reported in HEK-293 cells (Van der Hauwaert *et al.* 2014, Van Rooyen *et al.* 2018). Assays with 5α-reduced substrates indicated endogenous conversion yielding 5α,3α-reduced products in negative control samples; however, levels were negligible (<10%) in 11βHSD conversion assays in transfected cells.

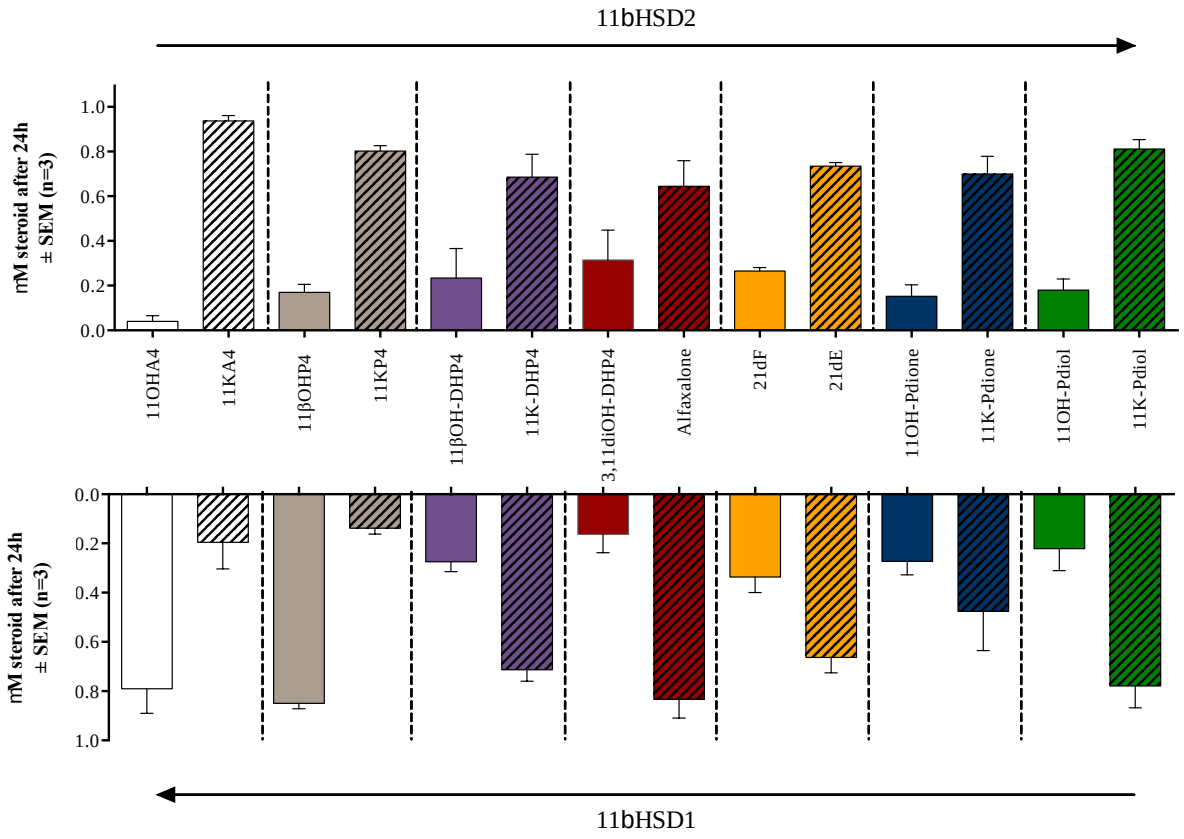

Figure 5. 2 Oxidation of C11-hydroxy steroids (solid bars) by 11βHSD2 (top; left to right) and the reduction of C11-keto steroids (banded bars) by 11βHSD1 (bottom; right to left). HEK-293 cells were co-transfected with 11βHSD2 and pCINeo or 11βHSD1 and H6PDH and the conversion 1 µM steroid substrate assayed after 24 hrs. Results are expressed as the mean ± SEM of three independent experiments performed in triplicate (n=3). Statistical analysis was carried out using an unpaired t test and showed all conversions p < 0001, except 11βHSD1 conversion of alfaxalone (p < 0.01), 11K-Pdione (p<0.001).

5.3.2 The interconversion of 11OHAST and 11KAST, and of 11OH-3αAdiol and 11K-3αAdiol by the 11βHSD

We showed the CYP17A1 conversion of 11OH-Pdiol and 11K-Pdiol —the C11-oxy backdoor pathway bottom tier steroids (Figure 5.1), yield 11OHAST and 11KAST which are substrates for the 17β-hydroxysteroid dehydrogenase isoforms converting the C11-oxy AST derivative to C11-oxy 3αAdiol and C11-oxy DHT steroid products (Figure 5.1). Although data generated using prostate cancer cell models suggest that the conversion of 11OHAST and 11KAST, as well as that

of 11OH-3αAdiol and 11K-3αAdiol is catalysed by 11βHSD2, these reactions have not, to date, been reported or shown explicitly. We therefore investigated the catalytic activity of both isoforms with the aforementioned substrates. The conversion of 1 µM 11OHAST to 11KAST by 11βHSD2 and the reverse reaction by 11βHSD1 was assayed in transiently transfected HEK-293 cells. A decrease in 11OHAST yielded negligible 11KAST (7.9 nM) after 24 h (Figure 5.3 A; right). In contrast, the 11βHSD1 catalysed reaction exhibited an ~85 % reduction in substrate with only 0.15 µM 11KAST remaining (Figure 5.3 B; left). Due to the poor ionisation of 11OH-3αAdiol and its low limit of detection, 10 µM substrate was used to investigate the interconversion of 11OH- and 11K-3αAdiol thus avoiding a false negative result. Only 11OH-3αAdiol was metabolised by 11βHSD2 yielding 1.68 µM 11K-3αAdiol (Figure 5.3 A; right) indicating a metabolic shift towards the keto derivatives since no 11K-3αAdiol was converted to 11OH-3αAdiol (Figure 5.3 B; right).

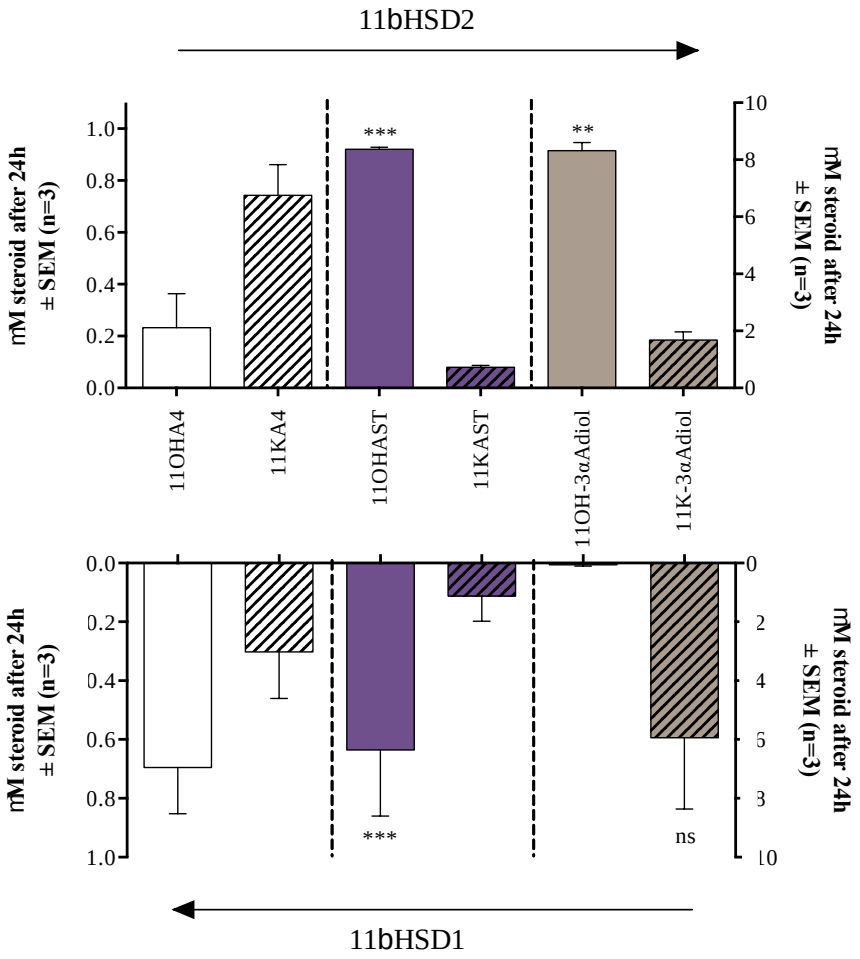

*Figure 5. 3 Interconversion of C11-oxy androgen metabolites by the 11βHSD isoforms. HEK-293 cells were co-transfected with (top) 11βHSD2 and pCINeo and the conversion of 1 µM 11OHA4 and 11OHAST, and 10 µM 11OH-3αAdiol assayed, and (bottom) 11βHSD1 and H6PDH and the conversion of 1 µM 11KA4 and 11KAST, and 10 µM 11K-3αAdiol assayed after 24 hrs. Results are expressed as the mean ±SEM of three or four independent experiments performed in triplicate (n=3). Statistical analysis was carried out using an unpaired t-test (**p ≤ 0.01; ***p ≤ 0.001).*

5.3.3 The metabolism of 11βOHP4 and 21dF in C4-2B cells

A pilot study was conducted investigating the metabolism of 11βOHP4 and 21dF in C4-2B cells transfected with CYP17A1. C4-2B cells, which expresses 11βHSD2 (Du Toit and Swart 2018), were transfected with CYP17A1 to engineer a C11-oxy C_{21} backdoor pathway cell model. Although analysis of the positive controls showed suboptimal transfection of CYP17A1 (data not

shown), the conversion of the 11OH-steroids to keto metabolites was nevertheless observed. After 96 h, 21dF —the 17α-hydroxylase product of 11βOHP4, was the major metabolite detected in the metabolism of 11βOHP4 together with low levels of keto-derivatives, 11KP4 (24 nM), 11K-DHP4 (8 nM) and 21dE (13 nM) (Figure 5.4 A). The metabolism of 21dF (Figure 5.4 B) yielded 21dE (0.26 µM) with negligible 11OHA4 and 11KA4 levels being detected.

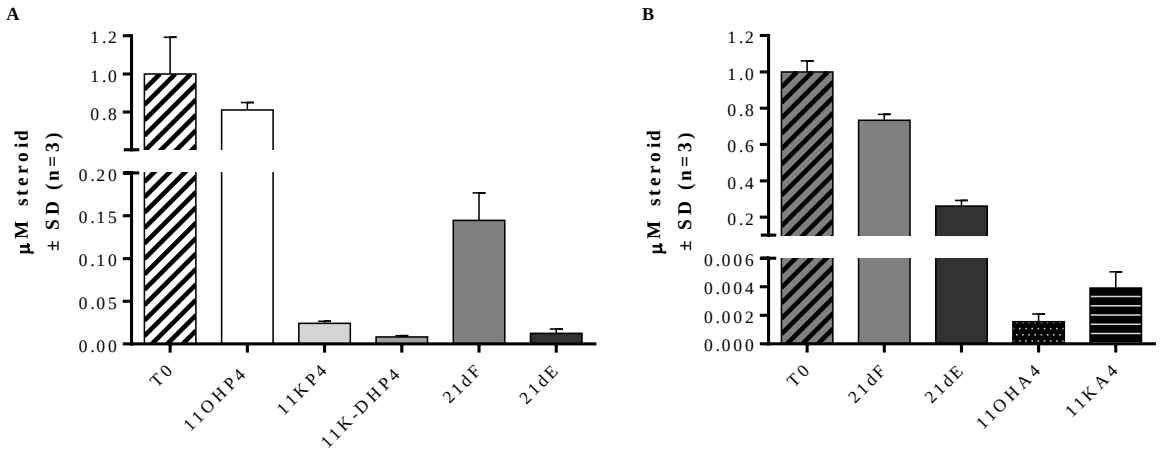

*Figure 5. 4 Metabolism of 11βOHP4 (A) and 21dF (B) by C4-2B cells transiently transfected with CYP17A1. Substrate conversion (1 µM) was assayed after 96 hrs. Results are expressed as the mean ±SD of an experiment performed in triplicate (n=3). Statistical analysis was carried out using an unpaired t-test (**p ≤ 0.01; ***p ≤ 0.001).*

5.4 Discussion

The action of glucocorticoids and C11-oxy C_{19} steroids is modulated by the 11βHSD1 and 11βHSD2 which, by oxidising or reducing the C11-oxy group, influences the physiological function of the steroid. In this study, we have provided *in vitro* evidence of 11βHSD activity in the C11-oxy backdoor and their potential role in androgen biosynthesis. We previously published the conversion of C11-oxy C_{21} steroids in the backdoor pathway catalysed by 5α-reductases, 3α-reductase type 3 (AKR1C2/3αHSD3) and the subsequent CYP17A1 conversion of C11-oxy C_{21} steroids to 11OHAST and 11KAST (Barnard *et al.* 2017, Van Rooyen *et al.* 2018, 2020). However, whether the C11-hydroxy and C11-keto metabolites are interconverted by the 11βHSD enzymes remained undetermined.

11βOHP4 and 11KP4 were interconverted by 11βHSD isozymes suggesting that, depending on the tissue expression, circulating 11βOHP4 in the presence of 11βHSD2 and absence of competing

enzymes, would be converted to its keto derivative and *vice versa in* the presence of 11βHSD1. However, 11α-hydroxyprogesterone (11αOHP4), corticosterone, dexamethasone and prednisolone inhibit 11βHSD2 activity while 11KP4, tetrahydrocortisone and 11-DHC inhibit 11βHSD1 (Bernal *et al.* 1980, Gent, du Toit, Bloem, *et al.* 2019). Data reported by Gent *et al.* (2019) showed that, *in vitro,* the conversion of 11OHA4, 11βOHP4 and 11OHT catalysed by 11βHSD2 occurred more readily than the reverse reaction of the keto derivative being catalysed by 11βHSD1 (Gent, du Toit, Bloem, *et al.* 2019).

These data suggested that the 11βHSD isozymes elicit a regulatory role beyond that of cortisol's inactivation in the protection of the MR, with the inhibitory activity of the aforementioned C11-oxy C_{21} steroids potentially contributing towards a tissue specific role in glucocorticoid activity towards the GR. In addition, the rapid conversion of 11βOHP4, 11αOHP4 and 11KP4 by 5α-reductases (SRD5A1 and SRD5A2) (Van Rooyen *et al.* 2018, Gent, du Toit, and Swart 2019) would suggest that 5α-reductases, yet again, act as gatekeepers of the backdoor pathway resulting in the production of 11βOH-DHP4, 11αOH-DHP4 and 11K-DHP4, respectively. Furthermore, the biosynthesis of 11OHP4 in the adrenal, expressing CYP17A1 and no 5α-reductases —would more likely direct the metabolism towards 21dF biosynthesis which upon peripheral metabolism be converted to 11OH-Pdione by SRD5A, or 21dE and subsequently 11K-Pdiol by 11βHSD2 (Figure 5.1).

The conversion of 11βOHP4 to 11KP4 and/or the subsequent reduction to 11K-DHP4 has previously been shown in LNCaP cells, endogenously expressing 11βHSD2 and SRD5A (Van Rooyen *et al.* 2018, Gent, du Toit, Bloem, *et al.* 2019). The SRD5A isozymes have been reported to be expressed in the same tissue as 11βHSD isoforms: 11βHSD1 and SRD5A are co-expressed in the liver (Russell and Wilson 1994, Hult *et al.* 1998) and gonads (Murphy 1981, Michael *et al.* 1997, Kayampilly *et al.* 2010), while 11βHSD2 and SRD5A are expressed in the gonads (ovary and testis) (Murphy 1981), skin, placenta and kidney (White *et al.* 1997) as well as prostate cancer cells and tissue (Albiston *et al.* 1994, Page *et al.* 1994, Iehlé *et al.* 1999, Dovio *et al.* 2009). In addition, expression and/or activity of the enzymes are elevated in the hyperandrogenic disorder, PCOS (Stewart *et al.* 1990, Fassnacht *et al.* 2003).

Recent reports expanding on the occurrence of the C11-oxy C_{21} steroids other than 11βOHP4 and 21dF are limited. Steroid profile analysis in neonates showed 11OHA4, 11KT and 11KP4 as the major steroids detected in the serum of both male and female with no 11βOHP4 detected (Du Toit

et al. 2018). Analysis of circulatory steroids in benign prostatic hyperplasia patients indicated higher C11-keto steroid levels which included 11KP4, 11K-DHP4, 11KT, 11KDHT, and 11KAST/11K-etio compared to their C11-hydroxyl derivatives with no 11βOHP4 or 11αOHP4 being detected. Furthermore, the 5α-reduced metabolite levels were also higher: DHP4 > P4, Pdione ≥ 17OHP4 and DHT > T together with 11KP4 levels exceeding that of 11K-DHP4 (Du Toit and Swart 2020). The data highlight the *in vivo* metabolism of the C11-oxy steroids favouring the keto metabolites, and while 11OHP4 is often not detected and its 5α-reduced metabolite not included in the analysis, its downstream products are.

We also showed the interconversion of 3,11diOH-DHP4 and alfaxalone, and 11OH-Pdione to 11K-Pdione, with less significant difference between the forwards and reverse reaction suggesting that the conversion of alfaxalone and 11K-Pdione to their C11-hydroxyl derivatives are more likely to occur compared to the reduction of the other C11-keto C_{21} steroids. Nevertheless, the metabolism of C11-oxy C_{21} steroids are driven towards the keto moieties.

These data suggested that the C11-oxy steroids together with their 11-keto derivatives should be included in clinical steroid analysis in order to obtain a better understanding of the prevalence and role of C11-oxy steroids in health and disease. It is clear that 11βHSD isozymes catalyse the interconversion of the C11-oxy C_{21} steroids in the top, middle and bottom tier as illustrated in the C11-oxy C_{21} backdoor pathway (Figure 5.1) and would, as such, contribute indirectly towards the production of C11-oxy C_{19} steroids. We previously showed the 17,20-lyase of 11OHPdiol and 11KPdiol, catalysed by CYP17A1, yields 11OHAST and 11KAST (Van Rooyen *et al.* 2020). In turn, 17βHSD and retinol-like hydroxysteroid dehydrogenase (RL-HSD) subsequently convert 11KAST to 11K-3αAdiol and 11KDHT contributing to the C11-oxy C_{19} pool. We have now conclusively shown the *in vitro* interconversion of 11OHAST and 11KAST catalysed by 11βHSD2 and 11βHSD1, respectively. Although 11OH-3αAdiol was converted to 11K-3αAdiol by 11βHSD2, under conditions in which the enzyme was saturated, this is unlikely to occur *in vivo* at physiological concentrations. Although 11OHAST and 11KAST are substrates for glucuronidation and would subsequently be excreted, 11KAST can also be converted to 11KDHT catalysed by 17βHSD.

Data presented show that 11βHSD1 and 11βHSD2 is capable of catalysing a number of C11-oxy steroids, with the oxidation reaction being more significant in the conversion of C11-oxy C_{21} steroids, however, enzyme expression levels would determine the flux through the pathway.

Kinetic parameters would be required to conclude on the metabolic fate and substrate affinity of SRD5A isozymes, 3α-dehydrogenases and CYP17A1 relative to 11βHSD for C11-oxy C_{21} steroids. It is nevertheless clear that 11βHSD1 and 11βHSD2 influence the production of C11-oxy C_{19} steroids in the C11-oxy backdoor pathway, not only affecting steroid levels in physiological homeostasis but also contributing towards therapeutic outcomes in the clinical manipulation of steroid levels in diseases and disorders.

Chapter 6

The transactivation of the progesterone receptor isoforms and the androgen receptor by C11-oxy steroids

6.1 Introduction

This study, thus far, has shown the metabolism of C11-oxy C_{21} steroid to potent androgens such as 11-ketodihydrotestosterone (11KDHT) which elicit a biological effect via its interaction with the androgen receptor (AR). In the previous chapter we addressed the role of the 11β-hydroxysteroid dehydrogenase (11βHSD) isozymes in the activation and inactivation of steroids as a regulatory mechanism for receptor interaction. However, the physiological role of 11β-hydroxyprogesterone (11OHP4), 11-ketoprogesterone (11KP4), 21-deoxycortisol (21dF) and 21-deoxycortisone (21dE) as well as their metabolic intermediates biosynthesised in the C11-oxy backdoor pathway, are largely unexplored.

Various physiological mechanisms are regulated by steroids interacting with specific receptors such as the activation of the nuclear steroid receptors, glucocorticoid- (GR), mineralocorticoid- (MR), estrogen- (ER), AR and progesterone receptors (PR) —by their specific steroid ligand. These receptors have been extensively studied in terms of their natural ligands but are also able to bind alternative steroid ligands, which can elicit a response similar to or different to the natural ligand. For example, aldosterone (ALDO), cortisol, progesterone (P4) and 11OHP4 can bind to the MR with high affinity but differ in terms of activity; ALDO and cortisol exhibits strong agonist activity while P4 and 11OHP4 exhibits weak agonist activity (Quinkler *et al.* 2002). In contrast, 11α-hydroxyprogesterone has a low binding affinity for the MR (Quinkler *et al.* 2002). Furthermore, in a rat study, 11KP4 was shown to have a higher affinity for the MR and exhibited greater mineralocorticoid activity compared to 11OHP4 (Galigniana *et al.* 2004).

The binding of potent androgens such as testosterone (T) and dihydrotestosterone (DHT) to the AR, even at low concentrations, induces a ligand-dependent conformational change and the subsequent translocation of the receptor-ligand complex to the nucleus where it binds to the DNA binding domain resulting in AR-regulated gene transcription. The AR regulates the expression of various target genes and play a regulatory role in the development and maintenance of reproductive tissue, and other biological processes. In addition, the AR plays a role in numerous androgen-

driven diseases and disorders. Prostate cancer (PCa) is classified as an androgen-dependent disease and is highly responsive to androgen deprivation therapy (ADT) or androgen receptor pathway inhibition (ARPI) therapy (Huggins and Hodges 1972). However, the cancer can re-emerge as castration-resistant prostate cancer (CRPC) which is sensitive to androgens as well as C_{21} steroids. CRPC exhibited changes in receptor and steroid enzyme expression profiles as well as steroid metabolism. AR expression is amplified in order to maintain AR signalling in the presence of low androgen levels (Knudsen and Scher 2009, Waltering *et al.* 2009). In addition, the expression of a mutated AR was reported in advanced metastatic CRPC cells and was bound by numerous steroids including P4, which induced transactivation of the mutated AR leading to receptor-regulated gene transcription (Veldscholte *et al.* 1992, Culig *et al.* 1993). Thus, the interaction of P4 with the mutated AR counteracts the ARPI therapy and consequently contributes to the progression of the disease. The decrease or lack of androgens due to ADT, results in increased C_{21} precursors biosynthesis signalled by luteinizing hormone (LH) via a negative feedback loop. More recently, a study reported the intracellular metabolism of C_{21} steroids yielding 20β-hydroxy-5α-dihydroprogesterone, in PCa cell lines which activated the AR and stimulated cell proliferation (Ando *et al.* 2018). Expression of the AR has also been reported in the testis (Leydig cells), mammary glands, heart (Ruizeveld De Winter *et al.* 1991, Takov *et al.* 2018), skin (sebaceous and sweat glands), placenta (Hsu *et al.* 2009), ovary (Horie *et al.* 1992, Suzuki *et al.* 1994), uterus (Mertens *et al.* 1996, Ito *et al.* 2002) and vagina (Ruizeveld De Winter *et al.* 1991), whilst controversial results are reported for AR expression in Sertoli cells (Ruizeveld De Winter *et al.* 1991, Boukari *et al.* 2009). Glucocorticoids and the GR have also been implicated in CRPC with an increase in GR expression reported in enzalutamide-resistant CRPC resulting in disease progression (Isikbay *et al.* 2014).

The expression profiles of nuclear receptors are complex with regards to endocrine-diseases and disorders with studies reporting increased expression or detection of steroid receptors in tissue which, under healthy conditions, do not express the receptor. The progression of PCa to CRPC have been attributed to the cancerous cells ability to utilise alternative steroids and/or pathways. Increased activity and enzyme expression of the backdoor and 11β-hydroxyandrostenedione (11OHA4) pathway as well as elevated C_{21}, C11-oxy C_{19} and C11-oxy C_{21} steroids have been reported in polycystic ovary syndrome (PCOS) (Marti *et al.* 2017), benign prostatic hyperplasia (BPH), PCa (Du Toit and Swart 2020) and CRPC (Dillard *et al.* 2008, Locke *et al.* 2008).

Cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) expression is elevated in CRPC, which was attributed to AR impairment with a positive correlation observed between CYP17A1 expression and abiraterone acetate concentrations in the VCaP prostate cancer cell line (Bremmer *et al.* 2014).

Disruption of the interaction between the prostatic epithelial and stroma cells is thought to result in the dysregulation of cell proliferation and differentiation, which subsequently lead to BPH (Niu and Xia 2009). In addition, both isoforms of the PR are expressed in stroma prostate cells, which also express the AR and ER, with increased expression linked to progression of PCa and CRPC (Mobbs and Liu 1990, Brolin *et al.* 1992, Bonkhoff *et al.* 2001, Latil *et al.* 2001, Yu *et al.* 2013). In contrast, it has also been suggested that the PR exert an inhibitory effect on PCa and BPH progression (Chen *et al.* 2017). Moreover, PCOS patients exhibits symptoms such as hirsutism, acne and oligomenorrhea which are a result of increased AR activity, overstimulated by the androgen excess (Apparao *et al.* 2002, Catteau-Jonard *et al.* 2008, Schüring *et al.* 2012).

This chapter also investigated the interaction of C11-oxy C_{21} steroids with the PR isoform. P4 activation of the PR plays an important role in regulation of female reproductive processes including ovulation; thus, when the system is disrupted the follicles cannot mature and cyst-like structures form, subsequently leading to subfertility. Furthermore, C11-oxy backdoor pathway enzymes including CYP17A1, 11βHSD, 5α-reductase, 3α-reductases and retinol-like hydroxysteroid dehydrogenase (RL-HSD) have been reported to be elevated in PCOS patients (Stewart *et al.* 1990, Rodin *et al.* 1994, Comim *et al.* 2013, Marti *et al.* 2017). Thus, C11-oxy C_{21} steroids can be metabolised in PCOS patients to yield C11-oxy backdoor pathway metabolites and/or potent androgen and subsequently alter the biological course via PR interaction.

It is, therefore, imperative that the C11-oxy C_{21} steroids, which are detected in tissue expressing the AR and/or PR, are investigated with regards to their ability to bind and activate these receptors, which subsequently leads to receptor regulated gene transcription.

6.2 Materials and methods

6.2.1 Materials

HEK-293 cells were purchased from the American Type Tissue Culture Collection (Manassas, VA, USA). Fetal bovine serum was supplied by Oxoid limited (Hampshire, England). Oxoid™ Phosphate Buffer Saline (PBS) tablets, penicillin-streptomycin (Pen-Strep), fetal bovine serum

(FBS), trypsin-EDTA and Pierce™ bicinchoninic acid (BCA) protein assay kits were purchased from ThermoFisher Scientific. XtremeGENE HP® DNA transfecting reagent were purchased from Roche Diagnostics (Mannheim, Germany). Dulbecco's modified Eagle's media (DMEM), phenol red-free DMEM and D-(+)-glucose were purchased from Sigma-Aldrich (St. Louis, USA). ViewPlate-96 (clear bottom, white 96-well microplate), TopSeal-A PLUS adhesive seal, BackSeal-96 adhesive seal, Neolite Reporter Gene assay system and promegastone (R5020) were purchased from PerkinElmer (MA, USA). The steroids were obtained from Sigma-Aldrich (St. Louis, USA), Steraloids (Newport, USA) and IsoScience (Pennsylvania, USA), and the deuterated steroid reference standards were purchased from Cayman Chemical company (Ann Arbor, Michigan, USA), CDN Isotopes Inc. (Augsburg, Bavaria, Germany) and Cambridge Isotopes (Andover, USA). Corning® CellBIND® Surface T75 flasks, 100 mm dishes and 96-well plates were purchased from Corning® Life Science (NY, USA). Nucleobond® Maxiprep DNA isolation kits were purchased from Macherey-Nagel (Duren, Germany). All general chemicals used were purchased from reliable scientific supply houses.

6.2.2 Plasmid constructs

The plasmids expressing the human PR isoforms (PR-A; pSG5hPR-A and PR-B; pSG5hPR-B) and the response element plasmid (pTAT-PRE-E1b-luc) were obtained from Dr. E. Kalkhoven (University Medical Center Utrecht, Utrecht, Netherlands) and Prof. G. Jenster (Erasmus University of Rotterdam, Netherlands), respectively. The human wild-type androgen receptor (pSG5/hAR) was obtained from Dr. H. Klocker (Innsbruck Medical University, Innsbruck, Austria, EU). The plasmid DNA was purified using the Nucleobond® Xtra Maxiprep DNA isolation kit (Machery-Nagal) as per instructions stipulated by the manufacturer. Due to the high homology shared between the DNA binding domain of steroid responses (Zilliacus *et al.* 1995), the PRE could be used for both PR and AR assays.

6.2.3 Transactivation studies via luciferase reporter promoter assays

HEK-293 cells were cultured to confluency in DMEM, pH 7.0, supplemented with 1.5 g/L $NaHCO_3$, FBS (10%) and 1% Pen-Strep (10 000 U/mL and 10 mg/mL, respectively) in Corning® CellBIND® Surface T75 flasks for at least 3 passages after which the cells were replated into 100 mm CellBIND dishes at 2 x 10^5 live cells/mL. Cells were co-transfected with the plasmids

expressing the steroid receptor (PR isoforms and AR), 0.9 µg, and the response element (9 µg), and incubated for 24 h. The transfected cells were replated into ViewPlate-96 at 5 x 10^4 live cells/mL using phenol red-free DMEM media supplemented with 10% dextran-stripped FBS and 1 % Pen-Strep. After 24 h incubation, the media was aspirated and cells treated with the steroid substrate (1, 10 and 100 nM, or 1 µM for the C11-oxy C_{19} steroids) prepared in unsupplemented phenol red-free DMEM and incubated for a further 24 h. The cells were treated with a vehicle control (ethanol, 0.1%), positive control (R5020 and P4 for PR and Mibolerone (Mib) and DHT for AR) and C11-oxy steroids. The luciferase reporter promoter assay was done directly after the incubation period; due to the influence of temperature on the assay, the assay plates (ViewPlate-96) and reagents were stored in a dark space at room temperature to equilibrate. The assay was conducted as stipulated by the manufacturer. Briefly, the reagents were mixed and added to the wells, the plates sealed using TopSeal-A and BackSeal-96. The plates were incubated in the dark for the specified time during which the plates were mixed via orbital mixing for 1 min using a Tecan Spark 10M multimode reader and subsequently centrifuged for 30 seconds to remove any bubbles formed. After 5 min, the luminescence was measured using the Tecan Spark 10M multimode reader.

6.2.4 Protein determination

In addition, a duplicate assay was performed in Corning® CellBIND® Surface 96-well plates for protein determination. Transfected cells described above were replated and assayed in parallel. After the final incubation step, the 96-well plates were centrifuged at 2000g for 10 min to collect all cells, the media aspirated, and cells resuspended in 20 µL passive lysis buffer (0.2% ($^v/_v$) triton, 10% ($^v/_v$) glycerol, 2.8% ($^v/_v$) tris–phosphate–EDTA, 0.9 M, and 0.288% ($^v/_v$) EDTA, 0.5 M). Plates were mixed via orbital mixing using the Tecan Spark 10M multimode reader and protein determined immediately using BCA protein assay kits.

6.3 Results

6.3.1 Transactivation of the androgen receptor by C11-oxy C_{21} steroid

The C11-oxy C_{19} steroids are prominent in disorders such as PCOS and 21-hydroxylase deficiency (21OHD). These steroids have been demonstrated to act via the androgen receptor, with the

androgenicity of 11OHT, 11KT and 11KDHT resembling that of T and DHT (Bloem *et al.* 2015). Elevated C11-oxy C_{21} steroids have also been reported in 21OHD (Turcu *et al.* 2015, Fiet *et al.* 2017). However, whether the C11-oxy C_{21} steroids and the C11-oxy backdoor pathway metabolites elicit a biological response via the human AR is still unclear. We assayed whether these steroids can induce transactivation of an AR responsive promoter in a pilot study using the Promega Luciferase Assay System; however, the data was unreliable as evident by numerous attempts and large error bars (data not shown). Due to the many steps in assaying luciferase activity, several factors could influence the success of the outcome including the transfection efficiency, normalisation to protein concentration, technical precision, etc. The Perkin Elmer neolite system was selected due to the lack of an aspiration step prior to the luminescence assay. Thus, assaying both adhering cells and cells which have lifted during the assay. In addition, precautionary steps were added to the assay to limit variation in results; adhesive seals were used to prevent evaporation, plates were centrifuged at numerous steps to collect all cells or remove bubbles which will skew luminescence reading and automated machinery was used to limit technical variations. We investigated whether 11OHP4, 11KP4, 21dF, 21dE, and 5α-reduced metabolites exhibit agonist activity via the AR at 1, 10 and 100 nM, and also included DHT and P4 as controls. Data are expressed as the mean (±SD) in order to show the large variation in results.

AR investigations presented 11OHP4 as an agonist for the human AR receptor and indicated an increase in activity with increasing steroid concentration (Figure 6.1 A-C). Only 11OHP4 demonstrated similar agonist activity to the natural AR ligand, DHT, which was significantly more than P4 and the other C11-oxy C_{21} steroids at 1 nM (Figure 6.1 A). At 10 nM (Figure 6.1.B) and 100 nM (Figure 6.1 C), 11OHP4 was able to induce transactivation via the AR to a significantly greater extent than DHT, the natural AR ligand in addition to the other steroids assayed, indicative of a super agonist. At 1 nM, agonist activity of 11OHP4 and DHT was similar and was significantly more than the other C11-oxy C_{21} steroids which showed no statistical difference. The other C11-oxy C_{21} steroids exhibited weak partial agonists activity with a response less than 25% of DHT at 1 nM, with P4 exhibiting 33% of DHT activity (Figure 6.1 A). The keto-derivatives, 11KP4, 11K-DHP4, 21dE and 11K-Pdione showed limited agonist activity via the AR at all concentrations tested, while 11OH-DHP4 and 21dF portrayed full agonist activity similar or greater than DHT at 10 and 100 nM (Figure 6.1 B and C). Although a previous study have reported

P4 to have no androgenic activity (Schindler *et al.* 2003), we demonstrate P4 to have partial agonist activity.

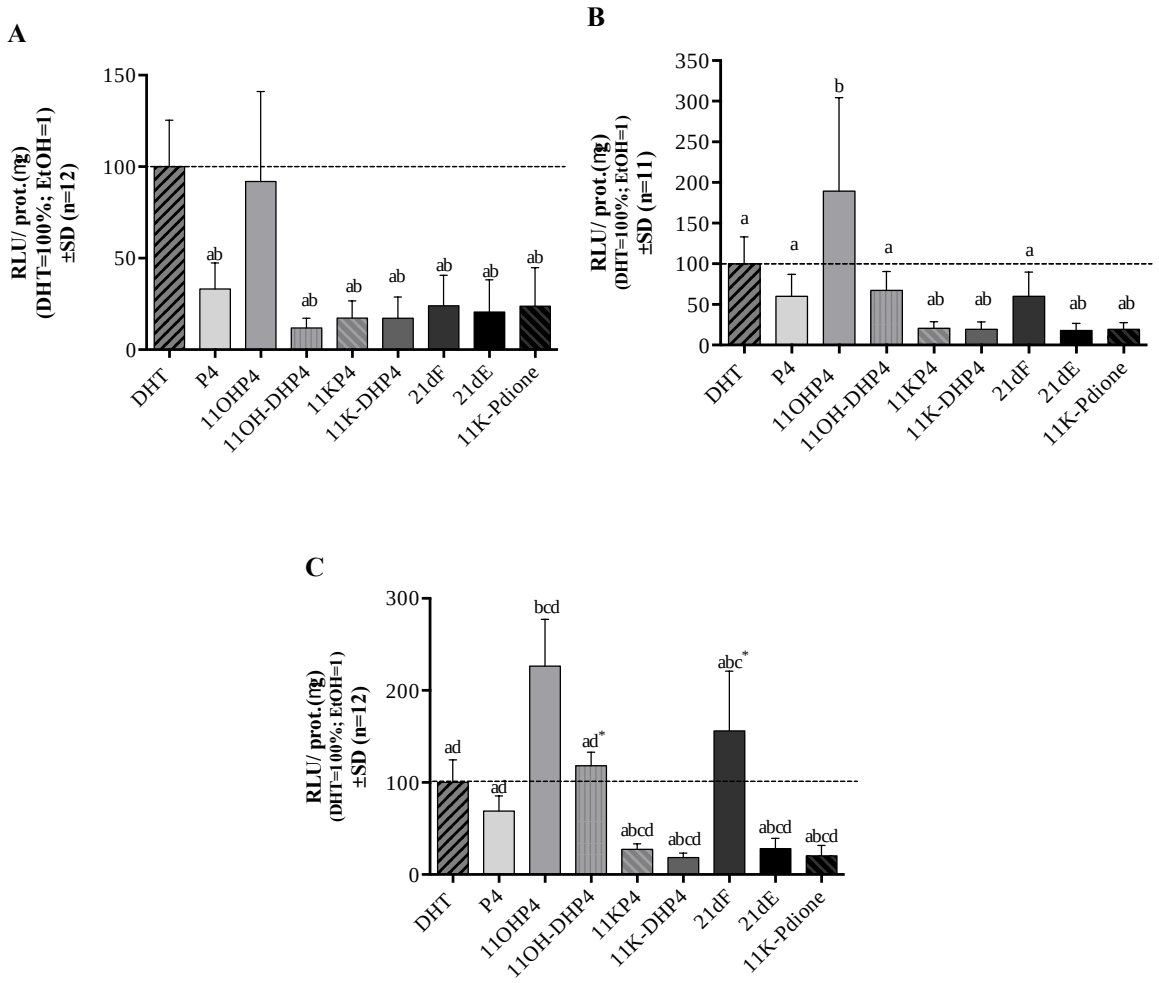

Figure 6. 1 Transactivation study investigating the agonist activity of C11-oxy C_{21} steroids towards the human AR. Steroids were assayed with 1 (A), 10 (B) and 100 nM (C) steroid substrate. Data represent three independent experiments performed in quadruplicate, except for 11OH-DHP4 which is representative of one independent experiment. Statistical analysis was done using one-way ANOVA followed by a Newman-Keuls post-test. Significant statistical differences were designated with reference to the steroid (a) 11OHP4, (b) DHT, (c) 11OH-DHP4 and (d) 21dF ($p \leq 0.0001$; c^*/d^*, $p \leq 0.05$).

6.3.2 Transactivation of the progesterone receptor isoform A and B by C11-oxy C_{19} and C11-oxy C_{21} steroids

Next, the ability of the C11-oxy C_{21} steroids to induce transactivation via the PR-A (Figure 6.2 left; A, C, E) and PR-B (Figure 6.2 right; B, D, F) were investigated at 1, 10 and 100 nM (Figure 6.2). Although 11OHP4 was able to induce transactivation via both PR isoforms, differences compared to P4, the natural ligand, were observed. Transactivation via PR-A was increased in response to increasing 11OHP4 concentrations, with the two highest concentrations assayed demonstrating significantly greater PR-A agonist activity than P4. No dose response effect relative to P4 was observed when comparing transactivation via PR-B. Thus 11OHP4 can be described as a super agonist and full agonist for the PR-A and PR-B, respectively (Figure 6.2). Unlike 11OHP4, 11OH-DHP4 exhibited partial agonist activity with the PR-B while none of the other C11-oxy C_{21} steroids showed more than 34 % activity relative to P4 (Figure 6.2 B). Similar to the AR, an increase in agonist activity was observed with increasing substrate concentration, specifically for the PR-A while the activity of the keto derivative towards the PR-B mostly remained low. Analysis of the 10 nM assays showed 21dF as a partial agonist of PR-A (Figure 6.2 C) while 11OH-DHP4 was exhibited the same activity towards both PR-A and PR-B (Figure 6.2 C and D) Lastly, the activity of 11OH-DHP4 as a full agonist was similar for the PR-A and PR-B at 100 nM (Figure 6.2 E and F). Unlike with the PR-B, 21dF exhibited partial agonist activity (Figure 6.2 E) while the remaining C11-oxy C_{21} steroids showed weak agonist activity.

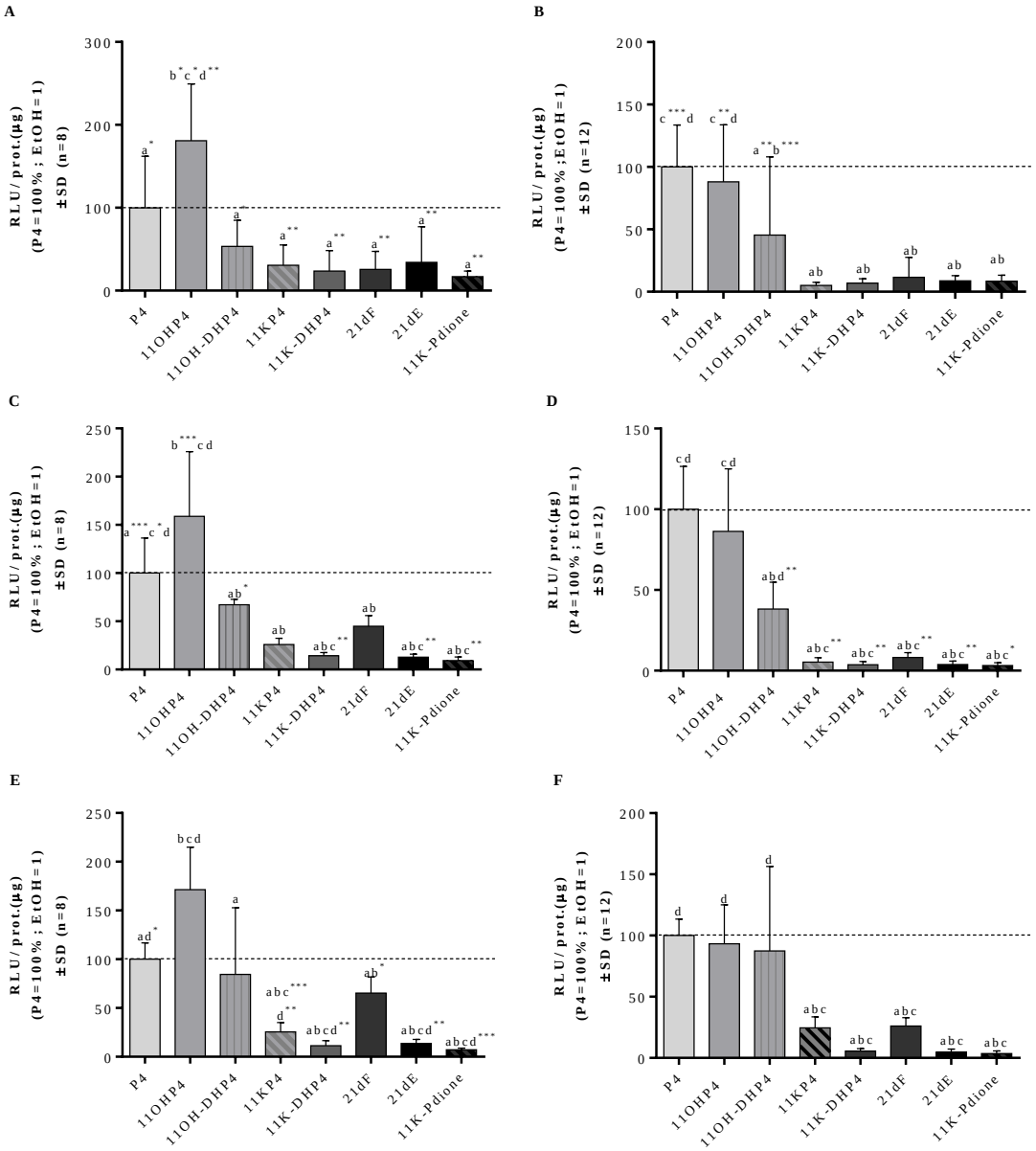

Figure 6. 2 Transactivation study investigating the agonist activity of C11-oxy C_{21} steroids towards the PR-A and PR-B. Interaction of 1 (A and B), 10 (C and D) and 100 nM (E and F) steroid substrate with the PR-A (left; A, C and E) and PR-B (right; B, D and F). Data represent three independent experiments performed in quadruplicate, except for 11OH-DHP4 which is representative of one independent experiment. Statistical analysis was done using one-way ANOVA followed by a Newman-Keuls post-test. Significant statistical differences were designated with reference to the steroid (a) 11OHP4, (b) P4, (c) 11OH-DHP4 and (d) 21dF with $p \leq 0.0001$ unless allocated otherwise (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

6.3.3 Interaction of C11-oxy C_{19} steroids with the progesterone receptor A and B

Investigations into the androgenicity of C11-oxy C_{19} steroids showed 11KT, and 11KDHT to have androgenic activity equal to that of T and DHT (Bloem *et al.* 2015). However, whether the C11-oxy C_{19} steroids could elicit a biological response via the progesterone receptor isoforms are unknown. This study showed no agonist activity by any of the C11-oxy C_{19} steroids via the PR-A or PR-B (Figure 6.3 A and B). The activity of 11KAST and 11KT with the PR-A differed statistically for the other C11-oxy C_{19} steroids but all were weak agonists (Figure 6.3 A).

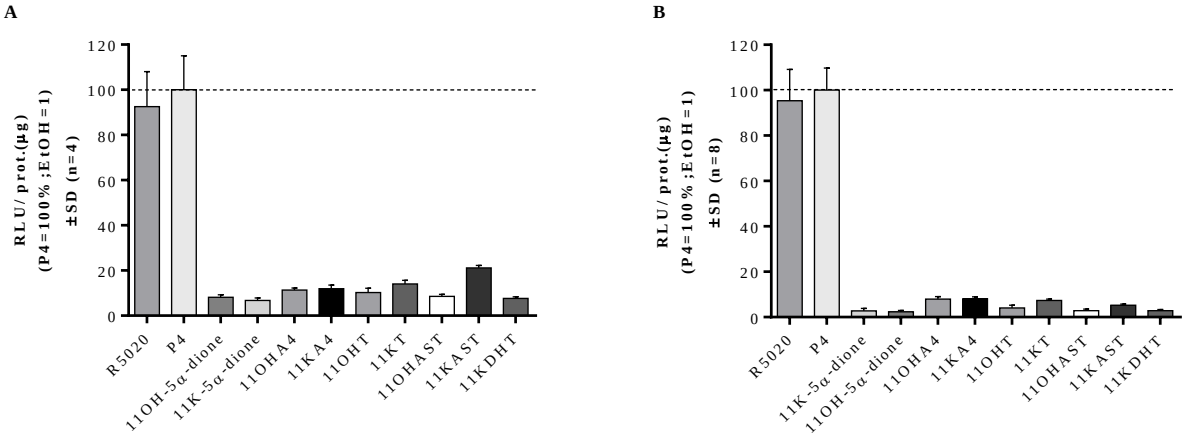

Figure 6. 3 Transactivation study investigating the agonist activity of C11-oxy C_{19} steroids towards the PR-A and PR-B. Steroids were assayed with 1 µM steroid substrate. Data pertaining to the PR-A and PR-B are representative of one and two independent experiments performed in quadruplicate, respectively. Statistical analysis was done using one-way ANOVA followed by a Newman-Keuls post-test (comparing all pairs of columns). All steroids differed statistically ($p \leq 0.0001$) from P4 and R5020 only (not indicated).

6.4 Discussion

Investigations into the ability of the AR and PR isoforms to induce transactivation in response to the C11-oxy C_{21} steroids will provide an insight into the physiological role of these steroids. Currently, *in vitro* and *in vivo* evidence of C11-oxy C_{21} steroid metabolism have been shown in PCa and BPH (Van Rooyen *et al.* 2018, 2020, Du Toit and Swart 2020); however, their physiological role in health and disease is unclear.

Analysis of transactivation by the AR in response to C11-oxy C_{21} steroids presented 11OHP4 as a full AR agonist, similar to DHT. Super agonist activity by 11OHP4 relative to the natural ligand DHT is already reached at a physiologically relevant concentration of 10 nM (Figure 6.1B). A broad physiological concentration range of 11OHP4 has been reported. In a study by Blumberg-Tick *et al.* (1991), 11OHP4 levels were reported as 10 nM in peripheral vein samples, 130 nM in adrenal vein samples and 115 nM in spermatic vein samples of 21OHD patients with testicular adrenal rest tumours (Blumberg-Tick *et al.* 1991). However, these levels were abnormally high compared to the majority of published results; 11OHP4 levels in 21OHD for basal and ACTH-stimulated was ~18 and 80 pM, respectively (Gueux *et al.* 1987, Fiet *et al.* 1989). Du Toit et al. (2020) reported, in increasing order, 11αOHP4, 11OHP4, 11KP4 and 11K-DHP4 in BPH tissue ranging from 11.1 – 538.3 ng/g as well as in circulation as unconjugated steroids ranging between 1-9 nM (Du Toit and Swart 2020). Moreover, 11OHP4 was undetected in healthy controls with levels in the low nmol/L range in 21OHD (Rege *et al.* 2019). Nevertheless, the transactivation data suggest that 11OHP4, even at low levels, could affect AR responsive gene transcription *in vivo*. Furthermore, 21dF showed partial agonist activity at the concentrations comparable with published *in vivo* levels reported as <1-35 nmol/L (Turcu *et al.* 2015, Fiet *et al.* 2017, Du Toit and Swart 2020) while at high nmol/L concentrations, 21dF was a full agonist. However, neither 11OHP4 nor 21dF have been detected *in vivo* at high levels. Thus, considering the *in vivo* levels and the data presented in this chapter, only 11OHP4 would significantly induce AR transactivation while 21dF will induce partial induction of transactivation. Androgens show higher androgenicity in its keto form compared to the hydroxyl derivative as well as its 5α-reduced moiety such as 11OHT and 11KT and subsequently 11KDHT. Interestingly, the opposite was observed for 11OHP4 and AR; transactivation via the AR induced by 11OH-DHP4 was significantly lower compared to 11OHP4 at all concentrations tested suggesting that reduction by SRD5A leads to an inactivation of the steroid. In addition, the AR activity also decreased once 11OHP4 was oxidised to 11KP4. Similarly, 21dF and 21dE activity correlated with 11OHP4 and 11KP4 with 21dE activity significantly lower at 100 nM compared to 21dF. The C11-oxy C_{21} steroids activity towards the AR resemble the glucocorticoid activity with the GR with higher agonist activity observed for steroid in it C11-hydroxyl form (Whorwood *et al.* 1992). Nevertheless, the remaining C11-oxy C_{21} steroids, 11OH-DHP4, 11KP4, 11K-DHP4, 21dE and 11K-Pdione —exhibited, at best, partial agonist activity at 1, 10 and 100 nM except for 11OH-DHP4 at 100 nmol/L which is unlikely to

occur in normal physiological conditions. The activation of the AR in endocrinological diseases such as PCa can have implications with regards to the disease prognosis. Furthermore, androgenic activity in CRPC was revived in the presence of low androgen levels by increasing the expression of the AR resulting in increased sensitivity towards androgens. The activation of the AR by low levels of 11OHP4 could possibly have the same effect in PCOS and CRPC patients where the AR expression is increased. Furthermore, the efficacy and potency as well as binding affinity of the steroids with AR remains unexplored. Steroids may elicit a biological influence by acting as competitor or by inducing transrepression.

Next, the transactivation of the PR-A and PR-B by the C11-oxy C_{21} steroids also showed a concentration dependent response with higher activities detected with increasing steroid substrate. 11OHP4 elicited full agonist activity towards both PR-isoforms with higher activity towards the PR-A. Interestingly, transactivation via the PR isoforms induced by 11OHP4, 11OH-DHP4 and 21dF is similar to the data generated for transactivation via the AR. Like for the AR, 11OH-DHP4 can be described as a full agonist at 100 nM for the PR-A and PR-B, increased promoter reporter activity induced by 21dF in a dose dependent manner was observed although transactivation via PR-B was slightly less, —and decrease PR activity in response to the 5α and C11 oxidised metabolites were demonstrated. Overall, the C11-oxy, C17-hydroxy steroids displayed weak or partial PR activity towards both PR isoforms. The two isoforms have different biological roles, which regulate the expression of isoform-specific genes or co-regulate the expression of others. Isoform expressions are comparable in healthy tissue such as the mammary gland; disruption of the ratio have been linked to cancerous condition including breast, endometrial, cervical and ovarian cancers (Richer *et al.* 2002, Esber *et al.* 2015). Furthermore, the biological outcome induced by the various receptors are influenced by the specific activity which is induced. The PR plays a vital role in regulating the menstrual cycle and subsequently influences fertility (Wetendorf and DeMayo 2014, Patel *et al.* 2015). PCOS is associated with subfertility or infertility; thus, it is important to investigate the C11-oxy C_{21} steroid interaction with the PR isoforms to elucidate whether the C11-oxy C_{21} steroids may promote dysregulation. Furthermore, elucidating the receptor interaction and subsequent biological responses induced by the C11-oxy C_{21} steroids may provide a better understanding of endocrine-disrupted diseases or provide possible therapeutic compounds.

Lastly, our research group has previously reported the androgenicity of C11-oxy C_{19} steroids (Bloem *et al.* 2015); due to the high homology shared between the ARE and PRE and the elevated C11-oxy C_{19} steroids in the above-mentioned clinical conditions, we investigated whether the C11-oxy C_{19} steroids can induce transactivation via the PR-A and/or PR-B. However, none of the C11-oxy C_{19} steroids investigated exhibited progestogenic activity suggesting that elevated C11-oxy C_{19} steroids will unlikely induce PR-regulated gene transcription.

This study has presented interesting results with the regards to activation of the AR and PR isoform, specifically by 11OHP4. However, large variation in the data was observed in the transactivation analysis by the steroids. Data generated using luciferase assay are notoriously inconsistent with numerous studies reporting results of either more than the typical triplicate independent experiment or numerous technical repeats with the purpose of excluding outliers (Ram *et al.* 1998, Chang *et al.* 1999, Pretorius *et al.* 2016). In this study, data were expressed as the mean and standard deviation instead of standard error of mean with the purpose of presenting large errors characteristic of the assay and in so doing subsequently highlighting limitations and shortfalls of the assay system. The variation in results were not only observed between independent experiments but also with large variation in the technical repeats; the maximum and minimum response normalised to protein for two independent experiments assaying P4 induction of PR-A, differ by 3 and 9-fold, respectively. This was also observed in the AR and PR-B for various steroids at various concentrations. In order to prevent variation in transfection which will subsequently influence the data, the HEK-293 cells were transfected prior to replating for experimental use and thus ensuring a homogenously transfected cell pool. The experimental error persisted regardless of the protocol followed. Furthermore, a pilot investigation was conducted in CV-1 cells stably transfected with the AR and ARE (Campana *et al.* 2016) which exhibit low endogenous receptor activity. In contrast to the assay conducted in HEK-293 cells, 11OHP4 showed no transactivation induction of the AR while the controls were successful and showed minimal variation in the data. HEK-293 cells have been reported to endogenously express GRα (Oakley *et al.* 1999) and is often considered negligible but the influence in this study was significant in impeding the assay outcome. In 2015, 21dF was shown to bind and activate the human GR (Pijnenburg-Kleizen *et al.* 2015) and the data presented in this chapter suggest a possible activation of the GR, with or without the AR and PR isoform, by 11OHP4, 11OH-DHP4 and 21dF. However, the findings are inconclusive as the receptors are able to interact with other nuclear receptors, bind the DNA as a monomer or

dimer, homozygous or heterozygous. Furthermore, substrates are incubated for 24 h during which endogenous enzymes, even at low expression levels, can convert the steroid substrate yielding metabolites which in turn can act as a false agonist.

In conclusion, the ability of C11-oxy C_{21} steroids to elicit a biological response via these steroid receptors remain uncertain. Although the data suggest possible receptor interaction of 11OHP4 and 21dF with either the GR with or without the AR and PR, the inconsistency in the results impede the assessment of the data. This chapter highlights the flaws in luciferase assay model cell systems which are currently still being used in many laboratories, questioning the validity of published data in the field of the steroid receptors, emphasising the need for new standards with regards to luciferase assays, with the focus on endogenous receptor activity as well as the metabolism of the steroid ligand. Further investigations are required to elucidate the role of C11-oxy C_{21} steroids in humans and their interactions with the steroid receptors.

Chapter 7

Manuscript to be submitted: Evidence of C11-oxy steroids in the serum and saliva of healthy male athletes.

7.1 Introduction

This study has, thus far, shown the biosynthesis of C11-oxy C_{21} steroids by CYP11B and their metabolism of these C11-oxy steroids to C11-oxy C_{19} steroids *in vitro* which, if the conversions occur *in vivo*, would contribute to the androgen pool in normal physiology and in hyperandrogenic conditions. Although *in vitro* enzymatic assays provide evidence of steroid conversion by a particular enzyme, these assays occur in a closed system in which the conversions are limited to the endogenous enzymes expressed in a single cell model and/or to transiently expressed enzyme. *In vivo,* however, steroids are transported throughout the periphery and are taken up in various endocrine tissue which express a wide array of steroidogenic enzymes that will compete and convert the substrates.

To date, no steroid profiles have been reported which include the full spectrum of steroids -studies rather focus on specific subsets of steroids. These, however, do not take into account all potential steroids in a particular metabolic pathway and base their deductions on limited information. Although some studies have reported circulating levels of 11β-hydroxyprogesterone (11OHP4), 21-deoxycortisol (21dF), 11-ketoprogesterone (11KP4) and/or 11-ketodihydroprogesterone (11K-DHP4) in clinical conditions (Turcu *et al.* 2015, Fiet *et al.* 2017, Du Toit and Swart 2020), the C11-oxy C_{21} steroid metabolites are rarely included in these analyses of circulating steroids. In the sporting community, steroid levels in athletes are monitored to identify steroids which would provide the athlete with a physical advantage. Although steroid levels of athletes, as well as those of patients are generally measured in urine and serum (Sottas *et al.* 2010, Martín-Escudero *et al.* 2015, Ponzetto *et al.* 2016), saliva has become an appealing alternative as the sampling is more dignified and more easily obtained from adults and children alike (Arruda *et al.* 2017, Crewther *et al.* 2018). Interestingly, anabolic-androgenic steroids currently considered by the anti-doping laboratories are limited to C_{19} steroids (World Anti-Doping Agency 2019a), regardless of reports showing the androgenicity of C11-oxy C_{19} steroids (Bloem *et al.* 2015, Swart and Storbeck 2015). Furthermore, having shown the conversion of C11-oxy C_{21} steroids to C11-oxy C_{19} steroids in this

thesis, it raises the question of whether these C11-oxy C_{21} steroids would serve as potential endogenous steroids administered in doping strategies. In addition to steroid hormones, athletes use hormone-stimulating compounds such as human chorionic gonadotropin (hCG) which stimulate androgen production without altering the urinary testosterone/epitestosterone ratio (Mareck *et al.* 2008, World Anti-Doping Agency 2019b). Studies have also shown that hCG induced testicular CYP11B1 expression (Yazawa *et al.* 2008) and increased 11OHP4 production (Combes-Moukhovsky *et al.* 1994) would result in higher C11-oxy steroid levels. It is, therefore, crucial to establish comprehensive steroid profiles in order to elucidate the levels of C_{19}, C_{21}, as well as the C11-oxy steroids in order to establish basal steroid levels enabling the identification of steroidogenic manipulation.

The manuscript reports the investigation into the levels of unconjugated steroids in serum and saliva of male athletes between the age of 18 and 35 in which the aim was to elucidate comprehensive steroid profiles. New UPC^2-MS/MS methods were developed in order to quantify the C_{19}, C_{21}, C11-oxy C_{19} and C11-oxy C_{21} steroids as well as epi-androgen derivatives. These analyses would simultaneously provide insights into recently described *in vitro* steroidogenic pathways.

Evidence of C11-oxy steroids in the serum and saliva of healthy male athletes.

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Abstract

Endogenous androgens are routinely measured in urine by GC-MS to monitor testosterone (T) misuse among athletes. However, only the classical androgens are included in doping control analysis. The adrenal C11-oxy C_{19} steroids as well as the C11-oxy C_{21} steroids which are metabolised to potent androgens in the periphery, places these steroids as potential compounds in doping strategies. This study developed an ultra-performance convergence chromatography-tandem mass spectrometry (UPC^2-MS/MS) method to analyse the full spectrum of C_{19} and C11-oxy C_{19} androgenic steroids including epitestosterone (epiT) in serum. Serum samples collected from 71 healthy male athletes, was analysed using the validated method. Further UPC^2-MS/MS analyses were subsequently carried out to determine C11-oxy C_{19} and C11-oxy C_{21} steroid levels as well as steroids of adrenal origin in serum and salivary samples. EpiT was detected above the LOQ (0.01 ng/ml) in 66 athletes at ~0.035 ng/mL (~0.12 nM) with T (~24.3 nM) being the dominant C_{19} steroid detected in circulation. In salivary samples T was however detected at very low levels, ~0.16 nM, while 5αDione (~8.7 nM) was detected in all athletes. The C11-oxy C_{19} steroids detected were 11β-hydroxyandrostenedione (~3.42 nM), 11β-hydroxytestosterone (~0.59 nM) and 11-ketotestosterone (~0.5 nM) with 11-ketoandrostenedione, the only C11-oxy C_{19} steroids detected in serum and saliva at comparable levels, ~0.9 nM. Limited C11-oxy C_{21} steroids were detected in serum samples. In salivary samples, 11β-hydroxyprogesterone, ~1.6 nM, was detected in 27 athletes, while cortisol (~5 nM) and cortisone (~14.8 nM) were detected in all test subjects whilst 11-dehydrocorticosterone (~1.3 nM) in 63 male athletes. Although steroid levels between age groups 18-25 and 26-35 years did not differ significantly, inter-individual steroid concentration and profiles differed

markedly thus supporting the comprehensive assessment of steroids levels not only in urine but also in serum and saliva.

Keywords: steroid profile, epitestosterone, ultra-performance LC-MS/MS; 11β-hydroxyandrostenedione (11OHA4); world anti-doping agency (WADA);

1. Introduction

Anabolic steroid abuse is an ongoing escalating practice in schools and health clubs worldwide with recreational weekend athletes and non-athletes wishing to perform better and/or to improve physical appearance and, more prominently, in sporting communities driven by competition and the desire to succeed [1,2]. Unfortunately, doping is associated with adverse side-effects spanning cardiovascular, hepatic, dermatological, reproductive and endocrinological as well as behavioural and neuropsychiatric complications [3]. The ever-changing trends in designer steroids used by professional athletes present challenges in the field of analytical chemistry. In the past decade immense strides have been made regarding the improvement in analytical procedures, particularly in the analysis of anabolic agents such as anabolic-androgenic steroids (AAS) by liquid chromatography mass spectrometry (LC-MS) in terms of more comprehensive steroid profiles, increased sensitivity and sample throughput. LC-MS is commonly used within clinical and referral laboratories world-wide, specifically tandem MS of LC-MS/MS ensuring high analyte specificity and speed of analysis.

Relating to the endogenous androgens or C_{19} steroids, only testosterone (T), androsterone (AST), dihydrotestosterone (DHT), androstenedione (A4), dehydroepiandrosterone (DHEA), androstenediol (A5) and their isomers and derivatives are currently included in the World Anti-Doping Agency (WADA) list of prohibited substances in the cohort of endogenous anabolic androgenic steroids administered exogenously [4]. In addition, only six C_{19} steroids (AST, T, epitestosterone (epiT), 5α-androstan-3α,17β-diol (3αAdiol), 5α-androstan-3β,17β-diol (3βAdiol) and etiocholanolone (etio), in their glucuronidated, sulfated and free forms, are analysed in urine for the athlete biological passport (ABP) in the monitoring of androgen levels over specific time periods, and are considered as markers indicating steroid doping [5]. In anti-doping testing, urinary T and epiT are both taken into account as the latter is produced in a fixed proportion to T during normal steroid biosynthesis and a ratio >4 is considered as evidence of doping by WADA [5]. Analyses of the T/epiT ratio is, however, complex with impaired conjugation resulting from uridine diphosphate glucuronosyltransferase (UGT) 2B17

deficiencies resulting in skewed analyses [6]. Other urinary steroid ratios which have been included in the ABP are AST/T (< 20), AST/etio, 3αAdiol/epiT and 3αAdiol/3βAdiol (> 2.4) [5,7]. In the conversion of T to DHT, the active androgen in steroidogenic tissue, the concentrations and ratios of androgens associated with the conventional and alternative pathways are generally considered (Figure 1; blue and purple), however, the passport profiles do not consider the contribution of 17α-hydroxyprogesterone's (17OHP4) metabolism to DHT production via the backdoor pathway (Figure 1; red).

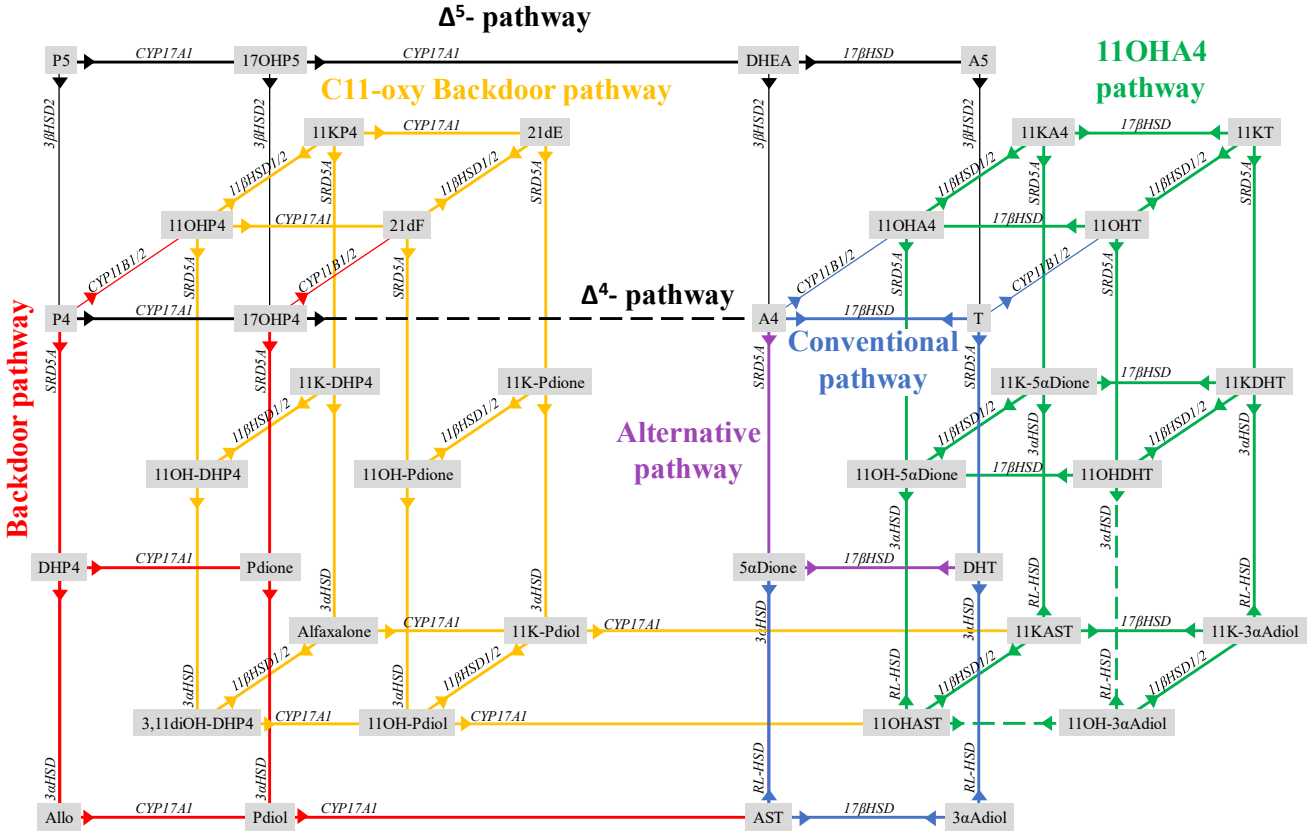

Figure 1: *Steroidogenic pathways leading to androgen production: the Δ[5]-androgen pathway (grey) yields androgen precursors, the backdoor (red), conventional (blue) and alternative (purple) pathway yield DHT and C11-oxy backdoor (orange) and 11OHA4 (green) pathway yield 11KDHT. The production of A4 in the Δ[4]- pathway is negligible in humans. The steroidogenic enzymes are shown on the arrows: cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1); cytochrome P450 11β-hydroxylase/ cytochrome P450 aldosterone synthase (CYP11B1/2); 5α-reductase (SRD5A); 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2); 11β-hydroxysteroid dehydrogenase type 1/type 2 (11βHSD1/2); 17β-hydroxysteroid dehydrogenase (17βHSD); 3α-hydroxysteroid dehydrogenase (3αHSD); retinol-like hydroxysteroid dehydrogenase (RL-HSD).*

Also contributing to the endogenous androgens are the C11-oxy steroids which have recently come to the fore [8–11] once more after having been overlooked for decades in the field of steroid research. C11-oxy C_{19} steroids and C11-oxy progesterones (C_{21} steroids), are produced in the adrenal gland with cytochrome P450 11β-hydroxylase (CYP11B1) catalysing the biosynthesis of 11β-hydroxyandrostenedione (11OHA4), 11β-hydroxyprogesterone (11βOHP4) and 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2 subsequently converting the C11 hydroxyl group to a keto (oxo) group in peripheral tissue and, to a lesser degree, in the adrenal. 11OHA4 is one of the major adrenal androgens with basal levels of 160 nM —2-fold higher than A4 with post-adrenocorticotropic hormone (ACTH) levels of 811 nM [12]. While the biosynthesis of 11OHA4 was initially considered as an inactivation mechanism in the conversion of A4 to T, our studies showed the peripheral conversion of 11OHA4 (Figure 1; green) to downstream C11-oxy metabolites yielding potent androgens such as 11-ketotestosterone (11KT), 11-ketodihydrotestosterone (11KDHT) and 11β-hydroxytestosterone (11OHT) [8,9,11]. In 2008, Yazawa *et al.* (2008) reported the androgenicity of 11KT, a major fish androgen, comparable to that of T [13] with our studies showing 11KT and its derivatives, 11OHT and 11KDHT, androgenic C11-oxy steroids in humans, able to bind and activate the androgen receptor capable of eliciting biological effects [14]. These C11-oxy C_{19} steroids have since been reported in healthy subjects [15,16] as well as in diseases such as prostate cancer and benign prostatic hyperplasia [10,17], and in clinical disorders such as polycystic ovary syndrome [18,19], 21-hydroxylase deficiency (21OHD) [20,21] and testicular adrenal rest tumours (TART) [22]. In addition to the metabolism of progesterone (P4) and 17OHP4 yielding DHT, we more recently showed that the metabolism of 11βOHP4, 11-ketoprogesterone (11KP4), 21-deoxycortisol (21dF) and 21-deoxycortisone (21dE) by peripheral steroidogenic enzymes yield C11-oxy C_{19} steroids via the C11-oxy backdoor pathway (Figure 1; orange) [9,23–25]. The metabolism of the C11-oxy C_{19} steroids and C11-oxy C_{21} steroids highlight seven potential pathways yielding potent androgens —three pathway yielding DHT and four yielding 11KDHT. It is therefore evident that these endogenous C11-oxy precursor steroids present the potential of being manipulated in the steroid doping arena. While research interest in the endogenous C11-oxy steroids is steadily on the rise, they have, to date, not been considered in anti-doping strategies given that the production of potent C11-oxy C_{19} steroids may, as in the case of T and DHT, enhance the performance of the athlete. Websites related to doping discussions confirm that the C11-oxy steroids are currently being administered to improve physical appearance and performance [26].

Further impacting on endogenous steroid levels is the administration of human chorionic gonadotropin (hCG), a glycoprotein hormone used to alter the urinary steroid profiles of athletes in doping strategies in which endogenous androgen biosynthesis is stimulated thus preventing changes in the T/epiT ratio [5,27]. The hormone has, however, been shown to also induce CYP11B1 and cyclic adenosine monophosphate (cAMP) in Leydig cells of immature mice [13] with a subsequent study [28] reporting increased 11βOHP4 levels in peripheral, gonadal and adrenal vein samples of 21OHD patients after the administration of dexamethasone and hCG. It is thus possible that exogenous hCG will induce CYP11B1 in athletes and shift steroidogenic pathways towards the production of C11-oxy C_{19} and C11-oxy C_{21} steroids which are currently not monitored.

Regardless of the evidence pertaining to the androgenicity of these C11-oxy C_{19} steroids and the impaired conjugation by glucuronidase and sulphatase enzymes due to the C11-oxy group [11,17], the exogenous administration of neither the C11-oxy androgens nor the C11-oxy progesterones or their metabolites are listed as prohibited substances. The inclusion of these C11-oxy steroids and their metabolites as prohibited substances (when administered exogenously) is critical in ensuring that these steroids be analysed and monitored, as they significantly contribute to the endogenous acitve androgen pool. The aim of this study was to investigate the occurrence of C11-oxy steroids in serum and saliva of 71 male athletes spanning two age groups, potentially highlighting target analytes relevant to anti-doping strategies. Our investigation initially focussed on the analysis of circulating C_{19} and C11-oxy C_{19} steroids and included epi-derivatives for which we developed and validated an ultra-performance convergence chromatography (UPC^2) tandem mass spectrometry (MS/MS) method. The use of supercritical fluid in UPC^2-MS/MS for steroids analysis offers higher resolution and selectivity for these structurally similar compounds when compared to LC-MS and is without the laborious sample preparation required for gas chromatography mass spectrometry (GC-MS). UPC^2-MS/MS merges the advantages of both LC-MS and GC-MS while eliminating limitations, thus providing an ideal analytical tool for routine high-throughput steroid analysis in clinical and doping-related scenarios for the detection and accurate quantification of steroids in the nM range. Once having established the levels of the epi-derivatives we analysed C_{19}, C11-oxy C_{19}, C_{21} and C11-oxy C_{21} steroids in both serum and saliva using a previously validated UPC^2-MS/MS method for the comparison of steroids in serum and saliva in the two matrices.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) was purchased from Oxoid limited (Hampshire, England). Lysozyme (muramidase, from hen egg white) was purchased from Roche Diagnostics (Mannheim, Germany), low maltose amylase from Wallerstein company, division of Travenol Laboratories, Inc. (Morton Grove, Illinois, USA) and albumen (egg powder) from BDH Chemicals Ltd (Poole, England). Oxoid™ Phosphate Buffer Saline (PBS) tablets were purchased from ThermoFisher Scientific. Analytical-grade methanol, formic acid, methyl *tert*-butyl ether (MTBE), and lipase (from *Candida cylindracea, Type VII)* —were purchased from Sigma-Aldrich (St. Louis, USA). Steroids were purchased from Sigma-Aldrich (St. Louis, USA), Steraloids (Newport, USA) and IsoScience (Pennsylvania, USA) and deuterated steroid reference standards from Cayman Chemical company (Ann Arbor, Michigan, USA), CDN Isotopes (Augsburg, Bavaria, Germany) and Cambridge Isotopes (Andover, USA). Waters UPC^2 Quality Control (QC) reference material containing trans-stilbene oxide, thymine, sulfamethoxazole and sulfamethizole was purchased from Waters corporation (Milford, USA). All other chemicals and reagents were purchased from reliable scientific supply houses.

2.2. Subjects

Following written informed consent, 71 healthy, active male participants from 27 countries were recruited for this study. Data collection took place between September and December 2018 during athlete pre-participation screening examinations. All participants had >8 years of high-level participation in their chosen sports (14.1 ± 5.4 yrs.), with sport-specific training for at least 8 hours per week (13.4 ± 5.6 hrs) across a range of 10 different sporting disciplines. Participant demographics (mean ± SD) were recorded for age (25.1 ± 4.8 yrs.), body mass (78.6 ± 15.0 kg), height (181.1 ± 11.4 cm), body surface area (1.93 ± 0.4 m^2) and body mass index (23.8 ± 2.7).

A complete medical history was collected by interview with each participant, to verify the exclusion criteria. Exclusion criteria: clinically significant acute or chronic disease; acute or chronic abuse of alcohol; drug abuse or excessive consumption of drugs (amphetamines, opiates, benzodiazepines, cannabinoids, barbiturates, cocaine, and tricyclic anti-depressive agents); pharmaceutical treatment including OTCs, vitamins, and/or supplements (except paracetamol for pain relief) 2 weeks prior to the study. All the participants underwent a complete medical screening procedure to ensure their healthy

state. All participants were in good health with no history of illness and were found eligible to participate in the present study. Written consent was provided by all the participants prior to their participation, and the study was approved by the Institutional Review Board of Weill Cornell Medicine-Qatar (1284681-2) and performed in accordance with the Declaration of Helsinki.

Each participant arrived at the laboratory following an overnight fast. Pre-exercise blood and saliva samples were collected in the morning between the hours of 08:00 and 09:30 am, thus preventing diurnal variations skewing data. Blood samples were immediately stored on ice after which samples were processed, serum collected and stored at -80 C° until extraction and analysis. Saliva samples were collected after participants thoroughly rinsed their mouth with water. A collection sponge placed under the tongue for a period of 3 minutes until fully saturated with saliva, was deposited into a collection container along with any excess saliva. Samples were stored at -80 C° until analysis. In order to avoid blood and other oral contaminations that may interfere with the assay, volunteers were requested to avoid dental work for 48 h prior to sample collection and to avoid teeth brushing 2 h prior to sample collection.

2.3. Sample preparation and steroid extraction

Saliva and serum samples were thawed on ice and saliva collected by centrifugation using a Hettich Universal Type 1200 centrifuge. Unconjugated steroids were extracted from serum and saliva using a liquid-liquid extraction method. Samples were spiked with internal standard mix, sonicated for 5 min in water at ~24 °C, ensuring that the handling of serum and saliva were identical, followed by the addition of 5 mL MTBE to 1 mL sample (5:1 ratio). The internal standard mix, 50 µL, which was added to all samples contained 15 deuterated steroids in deionised water (1 ng 4-pregnen-11β,17-diol-3,20-dione (2,2,4,6,6,21,21,21-D8) (21dF-d8), 1.5 ng 4-androsten-11β-ol-3,17-dione (2,2,4,6,6,16,16-D7) (11OHA4-d7), 5 ng 5α-pregnan-3,20-dione (1,2,4,5,6,7-D6) (DHP4-d6), 4-pregnen-11β,17,21-triol3,20-dione (9,11,12,12-D4) (Cortisol-d4), 5α-androstan-17β-ol-3-one (2,2,4,4-D4) (DHT-d4), 5α-androstan-17β-ol-3,11dione (16,16,17A-D3) (11KDHT-d3) and 4-androsten-17β-ol-3,11dione (16,16,17A-D3) (11KT-d3), 10 ng 4-pregnen-3,20-dione (2,2,4,6,6,17A,21,21,21-D9) (Prog-d9), 4-pregnen-17α-ol-3,20dione (2,2,4,6,6,21,21,21-D8) (17OHP4-d8), 4-androsten-17β-ol-3-one (1,2-D2) (T-d2) and 4-androsten-3,17-dione (2,2,4,6,6,16,16-D7) (A4-d7), 15 ng 5α-androstan-3α-ol-17-one (16,16-D2) (AST-d2), 20 ng 5α-androstan-3β-ol-11,17dione (9,12,12,16,16-D5) (11K-etio-d5), and 25 ng 5α-androstan-3α,17β-diol (16,16,17A-D3) (3αAdiol-d3) and 5-androsten-3β-ol-17-one (2,2,3,4,4,6-D6) (DHEA-d6)).

The samples were vortexed for 15 min at 1700 rpm using a MRC Multi-Tube Vortexer and placed at -80 °C. The organic phase was removed from the frozen aqueous phase and dried under nitrogen gas at 40 °C. Samples were resuspended in 75 µL 100 % analytical-grade methanol, transferred to a glass insert after which 75 µL deionised water was added. All samples were subsequently stored at -20 °C prior to MS analysis.

In the analysis of serum and saliva samples of the athletes the C_{19} and C11-oxy C_{19} steroid levels, which included the epiT, epiAST, epi11KAST and 11OHAST were firstly quantitated using the *UPC²-MS/MS* method described below. Once these steroid levels were ascertained the C11-oxy steroids which included the C_{19} and C11-oxy C_{19} steroids together with the C_{21} and C11-oxy C_{21} steroids as well as the glucocorticoids and mineralocorticoids produced by the adrenal were analysed and quantitated using a recently developed method which enabled the separation of these steroids in a single chromatographic step [29].

2.4. C_{19} and C11-oxy C_{19} UPC²-MS/MS method development and validation

2.4.1. *UPC²-MS/MS conditions*

Steroid analysis was performed with an ACQUITY UPC² system coupled to a Xevo TQ-S triple quadruple mass spectrometer (Waters Corporation, Milford, USA). Steroids were separated on a Viridis® Supercritical Fluid Chromatography ethylene-bridged hydrid (BEH) 2-ethylpyridine (2-EP) column (3.0 x 100 mm, 1.7 µm) fitted with a VanGuard™ pre-column (2.1 x 5 mm, 1.7 µm) (Waters corporation, Milford, USA).

The following MS conditions were used: column temperature set to 60 °C, automated back pressure regulator (ABPR) to 1800 psi, capillary voltage of 3.8 kV, source temperature 150 °C, desolvation temperature and gas flow rate were set to 500 °C, and 900 L/h, cone gas 150 L/h, nebuliser gas flow set to 7 bar and collision gas flow 0.15 mL/min. Post-column infusion of 1% formic acid in methanol was pumped at a constant flow rate of 0.2 mL/min.

The chromatographic separation of the C_{19} and C11-oxy C_{19} reference standards was achieved using the elution gradient described in Supplementary Table 1. The wash and re-equilibration step proved sufficient in preventing carryover of any analyte at the given concentrations.

All steroids were analysed in multiple reaction monitoring (MRM) mode using positive electrospray ionisation (ESI+) mode, with MS parameter settings together with quantifiers and qualifiers shown in Supplementary Table 2 and the LOD and LOQ values in Supplementary Table 3. Selectivity was ensured

by chromatographically separating any interferences from the matrices or other compounds that crosstalk due to similarities between molecular ion *m/z* and/or fragment ion *m/z* in the region of elution of each compound. In instances where the chromatographic separation of the compounds was not possible, MRM's which do not crosstalk were selected. Response factors were calculated for steroid impurities detected in steroid standards, since impurities will contribute to steroid levels resulting in an underestimation or an overestimation, depending on impurity.

2.4.2. Standard solutions and calibration samples

Steroid standards, 2 mg/mL, were dissolved in absolute ethanol and used to prepare five steroid master mixes, 0.1, 10, 1000, 2000, 5000 ng steroid /mL, in 50% methanol. The steroid calibration range (0.0005-1000 ng/mL) was prepared by spiking dextran-coated charcoal stripped foetal bovine serum (DCC-FBS) or a surrogate saliva matrix, 1000 µL, with an appropriate volume of the steroid working mix containing the relevant steroids. Calibration samples, 19, including a zero (extracted matrix and IS), were prepared at the following concentrations: 0.0005, 0.00125, 0.0025, 0.005, 0.0125, 0.025, 0.05, 0.125, 0.5, 1.25, 5.0, 12.5, 50.0, 125.0, 250.0, 500.0, 750.0, and 1000.0 ng/mL. Internal standard (IS) was added and the samples extracted as described in section 2.3. Injection volume for all samples was 2 µL and blank (50 % methanol) 5 µL. Waters UPC^2 Quality Control (QC) reference material containing trans-stilbene oxide, thymine, sulfamethoxazole and sulfamethizole was interspersed with samples during analysis and used to monitor chromatographic performance. A calibration curve was generated by plotting the ratio of the quantifier peak area and the IS peak area over the concentration using a linear regression equation and a $^1/_x$ weighting scheme and used to determine the limit of detection (LOD), limit of quantification (LOQ), calibration range (ng/mL), and linearity (r^2) for each steroid (Supplementary table 3).

2.5. Statistical analysis

GraphPad Prism 6 (GraphPad Software, Inc., CA, USA) was used for statistical analysis and for figure compilation. Data are expressed as the median with interquartile ranges (IQR). Multiple t test analysis of serum and saliva showed no statistical difference between normal (n=48; BMI, 18.5-24.9) and overweight (n=21; BMI, 25-32) steroid levels or between ethnic groups (Arab (n=38), Asian (n=2), Black (n=19) and Caucasian (n=12)).

3. Results

3.1. UPC² separation and method validation

The chromatographic separation of the twenty-five C_{19} and C11-oxy C_{19} steroids was achieved in 8.5 min (Figure 2) using the gradient described in Supplementary table 1. Included in the method were 10 deuterated standards and four UPC² QC reference compounds (Supplementary table 2). All of the steroids which posed a problem with regards to crosstalk, were successfully separated and exhibited good resolution. The crosstalk peak of a steroid with similar retention time is visible in the windowed channel of the steroid with which the steroid crosstalk resulting in two or more peaks.

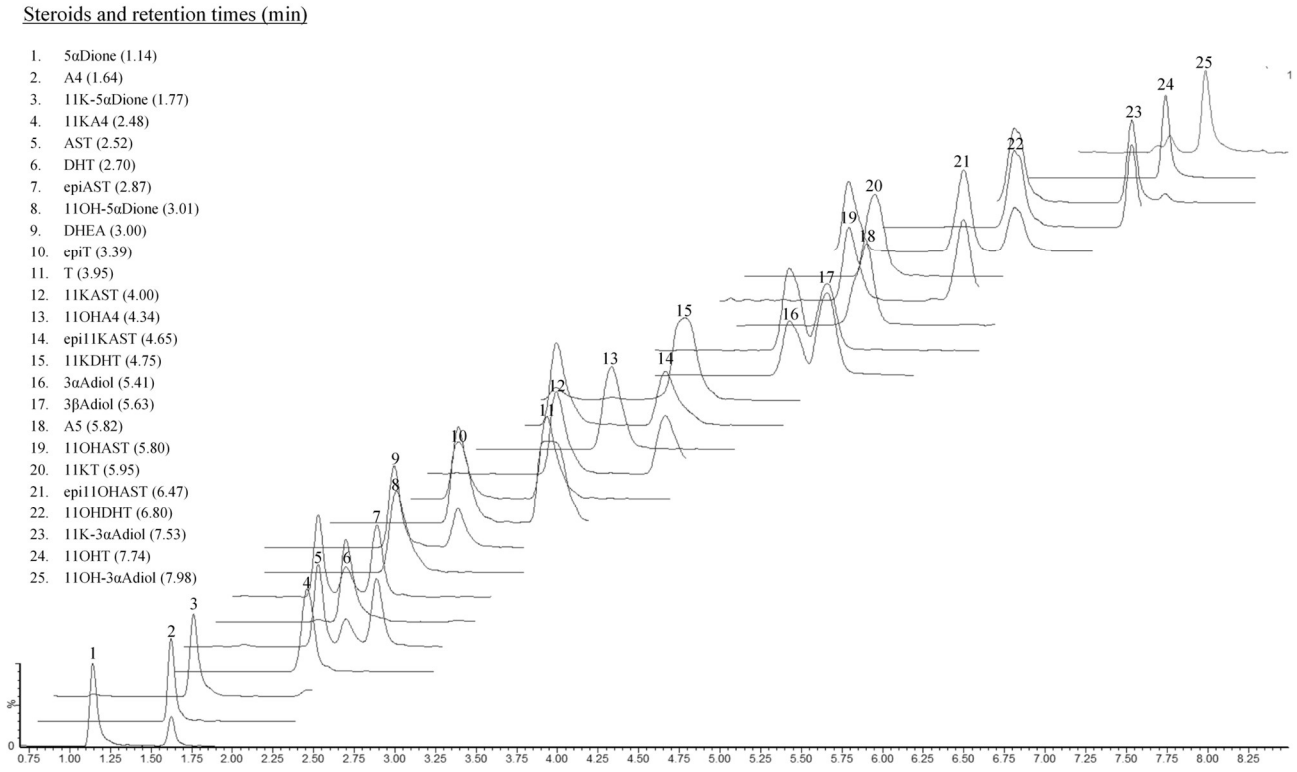

Figure 2: *Chromatogram of 25 C_{19} and C11-oxy C_{19} steroid reference standards generated by UPC²-MS/MS analysis.*

Analysis of steroidal crosstalk interference showed that a number of steroids crosstalk with other steroids depicted in Figure 3 with injected steroid listed to the left and crosstalk interference listed above. For example, injecting 5αDione, which eluted at 1.14 min, also elicited a peak at 1.14 min in the channel of DHT, DHEA, 11OHAST and D5-11KAST. However, in this instance the crosstalk peak does not alter the resolution of the steroids due to their elution at other retention times. In the case of AST, DHT and epiAST (Figure 2; peak #5, 6 and 7) which all crosstalk and elute close to each other, all three peaks are

visible in the other channels and required gradient manipulation during development to ensure separation. In cases where the crosstalk could not be resolved by altering the ion fragment MS properties, the gradient was adjusted by either manipulating the solvent make-up or time range of the chromatographic step. It is therefore crucial to know the precise retention time of a steroid in order to prevent quantification of the interference peaks.

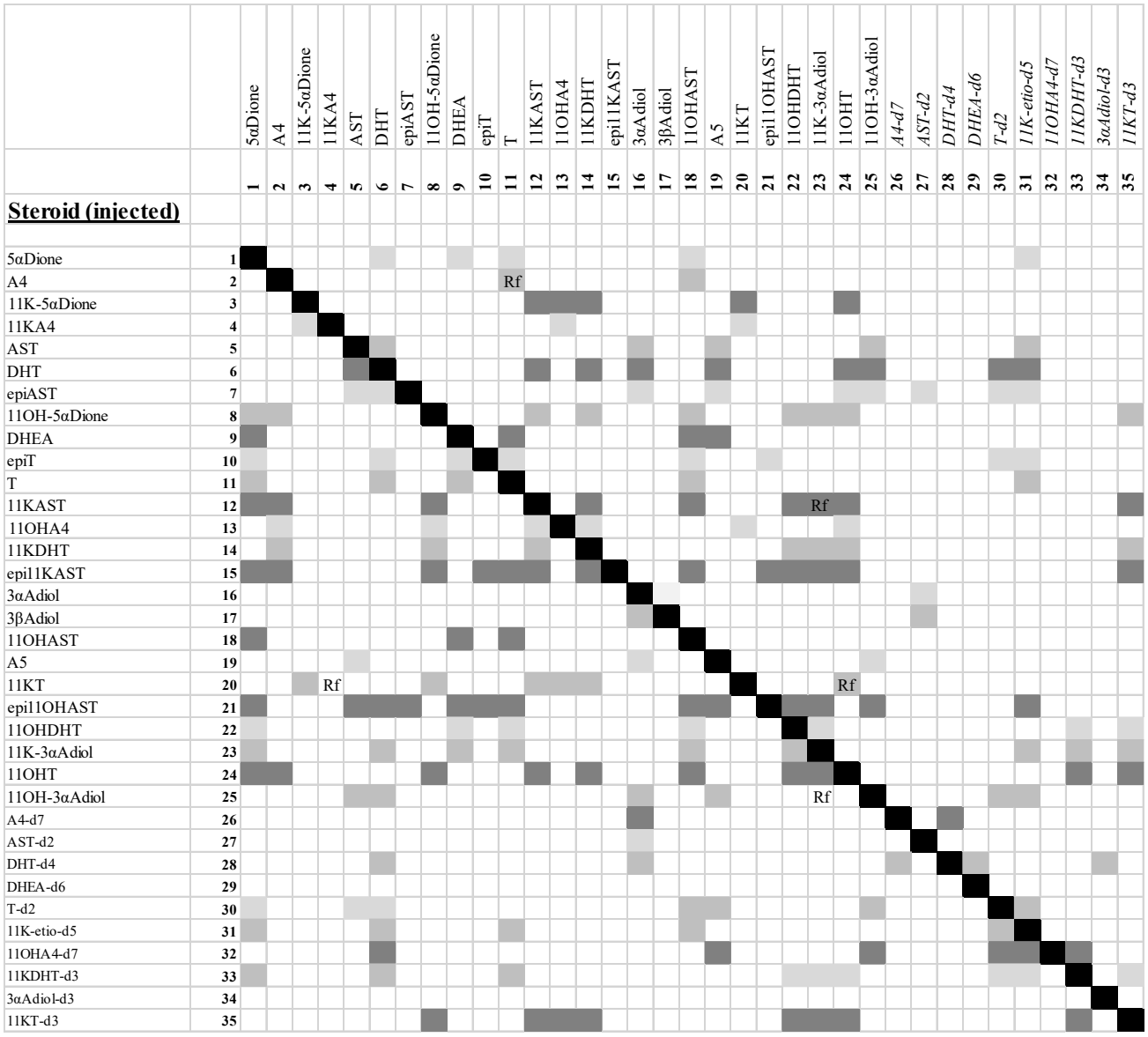

Figure 3*: Steroid crosstalk in channels. Solid blocks (tones of grey) in the row of the steroid standard injected (left) represent a crosstalk peak detected in the channel of the analytes (above). Instances in which response factors were applied in analyses are indicated as Rf: A4 standard contains T, 11KAST and 11OH-3αAdiol standards contain 11K-3αAdiol and 11KT standard contains traces of both 11KA4 and 11OHT.*

The UPC^2-MS/MS method used to simultaneously analyse the 52 steroids [29], was validated using 1 mL serum and saliva matrices; the steroids are listed in Table 1 with the LOD, LOQ, calibration range and r^2 value calculated for each steroid in serum and saliva. Differences in the LOD and LOQ of a steroid in the different matrices highlights the influence of the matrix on the ionisation of a steroid resulting in either ionisation suppression or enhancement which subsequently influences the LOD and LOQ. Steroid compounds can also form adducts with compounds such as sodium and potassium which are both present in the PBS-based saliva surrogate matrix used for method validation. Hence, the composition of a matrix may improve extraction of a particular steroid in said matrix compared to another as well as influence the viscosity of the sample droplet which makes it less volatile. Steroids may have different interactions with the matrices thus resulting in independent alterations in the LOD and LOQ values.

Table 1

Steroid standards used in the UPC^2-MS/MS analysis. IUPAC names, trivial names and abbreviations are depicted with their molecular weight (Mr). Method validation was specific to serum and saliva as matrices showing the limit of detection (LOD), limit of quantification (LOQ), calibration range (ng/mL) and linearity (r^2) in each matrix.

Compounds				Serum (1 mL)				Saliva (1 mL)			
IUPAC name	Trivial name	Abbreviation	Mr	LOD* ng/mL	LOQ* ng/mL	Calibration range†	r^2 †	LOD* ng/mL	LOQ* ng/mL	Calibration range†	r^2 †
5α-androstan-3,17-dione	androstanedione	5αDione	288.4	0.125	0.5	0.5 - 1000	0.9990	0.125	0.5	0.5 -750	0.9992
5α-pregnan-3,20-dione	dihydroprogesterone	DHP4	316.48	1.25	5.0	5.0 - 1000	0.9990	0.5	1.25	1.25- 1000	0.9973
4-pregnen-3,20-dione	progesterone	P4	314.47	0.125	0.125	0.125 - 750	0.9995	< 0.125	0.125	0.125 - 1000	0.9962
4-androsten-3,17-dione	androstenedione	A4	286.4	0.05	0.125	0.125 - 1000	0.9984	0.125	0.5	0.5 - 750	0.9980
5α-androstan-3,11,17-trione	11-ketoandrostanedione	11K-5αDione	302.41	0.5	1.25	1.25 - 500	0.9946	0.5	1.25	1.25 - 250	0.9970
5α-pregnan-3,11,20-trione	11-keto-dihydroprogesterone	11K-DHP4	330.46	0.5	1.25	1.25 - 500	0.9970	0.125	0.5	0.5 - 500	0.9962
4-androsten-3,11,17-trione	11-ketoandrostenedione	11KA4	300.4	0.125	0.5	0.5 - 125	0.9988	0.125	0.5	0.5 - 125	0.9998
5α-androstan-3α-ol-17-one	androsterone	AST	290.4	1.25	5.0	5.0 - 125	0.9974	5.0	12.5	12.5.0 - 1000	0.9955
4-pregnen-3,11,20-trione	11-ketoprogesterone	11KP4	328.45	0.05	0.125	0.125 – 125	0.9957	< 0.125	0.125	0.125 - 1000	0.9997
5α-pregnan-17α-ol-3,20-dione	pdione	Pdione	332.48	12.5	12.5	12.5-500	0.9272	12.5	12.5	12.5 - 500	0.9811
4-pregnen-21-ol,3,20-dione	deoxycorticosterone	DOC	330.46	1.25	5.0	5.0 - 125	0.9806	1.25	5.0	5.0 - 125	0.9943
5α-androstan-17β-ol-3-one	dihydrotestosterone	DHT	290.44	0.05	0.125	0.125 - 50	0.9969	0.05	0.125	0.125 - 125	0.9971
5-androsten-3β-ol-17-one	dehydroepiandrosterone	DHEA	288.4	0.5	1.25	1.25 - 1000	0.9994	0.5	1.25	1.25 - 500	0.9997
5α-pregnan-3β-ol-20-one	Epi-allopregnanolone	epiAllo	318.5	0.125	0.5	0.5 - 1000	0.9991	1.25	1.25	1.25 - 500	0.9993
5α-androstan-11β-ol-3,17-dione	11β-hydroxyandrostanedione	11OH-5αDione	290.4	1.25	5.0	5.0 - 1000	0.9994	1.25	5.0	5.0 - 1000	0.9992
5-pregnen-3β-ol-20-one	pregnenolone	P5	316.48	1.25	5.0	5.0 - 750	0.9997	1.25	5.0	5.0 - 750	0.9991
5α-pregnan-11β-ol-3,20-dione	11β-hydroxy-dihydroprogesterone	11βOH-DHP4	332.48	1.25	5.0	5.0 - 750	0.9990	5.0	12.5	12.5 - 1000	0.9989
4-pregnen-17α-ol-3,20-dione	17α-hydroxyprogesterone	17OHP4	330.47	1.25	1.25	1.25 - 1000	0.9986	5.0	5.0	1.25 - 1000	0.9984
4-androsten-17β-ol-3-one	testosterone	T	288.4	0.5	1.25	1.25 - 125	0.9956	< 0.5	0.5	0.5 - 125	0.9994

5α-androstan-3α-ol-11,17-dione	11-ketoandrosterone	11KAST	304.4	0.5	1.25	1.25 - 1000	0.9990	1.25	5.0	5.0 - 1000	0.9998
4-androstene-11β-ol-3,17-dione	11β-hydroxyandrostenedione	11OHA4	302.4	0.05	0.125	0.125 - 500	0.9960	0.125	0.5	0.5 - 750	0.9987
5α-pregnan-17-ol-3,11,20-trione	11-keto-pdione	11K-Pdione	346.5	1.25	5.0	5.0 - 750	0.9992	1.25	5.0	5.0 - 1000	0.9991
5α-pregnan-11α-ol-3,20-dione	11α-hydroxy-dihydroprogesterone	11αOH-DHP4	332.5	0.5	1.25	1.25 - 750	0.9999	0.5	1.25	1.25 - 1000	0.9989
5α-pregnan-3α-ol-11,20-dione	alfaxalone	Alfaxalone	332.48	0.5	1.25	1.25 - 750	0.9967	1.25	1.25	1.25 - 500	0.9970
5α-androstan-17β-ol-3,11-dione	11-ketodihydrotestosterone	11KDHT	304.4	0.125	0.5	0.5 - 750	0.9984	0.5	1.25	1.25 - 500	0.9967
4-pregnen-11β-ol-3,20-dione	11β-hydroxyprogesterone	11βOHP4	330.46	0.5	1.25	1.25 - 500	0.9996	0.005	0.0125	0.0125 - 500	0.9993
4-pregnen-21-ol-3,11,20-trione	11-dehydrocorticosterone	11-DHC	344.45	0.05	0.125	0.125 - 500	0.9997	0.05	0.125	0.125 - 500	0.9999
4-pregnen-16α-ol-3,20-dione	16α-hydroxyprogesterone	16OHP4	330.46	0.05	0.125	0.125 - 500	0.9993	0.05	0.125	0.05 - 500	0.9989
5α-androstan-3α,17β-diol	androstanediol	3αAdiol	292.46	1.25	5.0	5.0 - 750	0.9988	1.25	5.0	5.0 - 500	0.9989
4-pregnen-17-ol-3,11,20-trione	21-deoxycortisone	21dE	344.44	0.125	0.125	0.125 - 500	0.9990	0.05	0.125	0.125 - 500	0.9993
4-pregnen-11α-ol-3,20-dione	11α-hydroxyprogesterone	11αOHP4	330.46	0.5	1.25	1.25 - 500	0.9992	0.125	0.5	0.5 - 1000	0.9972
5α-androstan-3α,11β-diol-17-one	11β-hydroxyandrosterone	11OHAST	306.44	5.0	12.5	12.5 - 750	0.9993	5.0	12.5	1.25 - 750	0.9990
5α-pregnan-3α,17α-diol-20-one	Pdiol	Pdiol	334.48	1.25	12.5	12.5 - 1000	0.9995	5.0	12.5	12.5 - 750	0.9988
5-androsten-3β,17β-diol	androstenediol	A5	290.4	1.25	5.0	5.0 - 750	0.9999	1.25	5.0	5.0 - 500	0.9989
4-androsten-17β-ol-3,11-dione	11-ketotestosterone	11KT	302.41	0.5	0.5	0.5 - 1000	0.9960	0.125	0.5	0.0125 - 500	0.9994

4-pregnen-17α,21-diol-3,20-dione	11-deoxycortisol	11-deoxycortisol	346.46	0.125	0.5	0.5 - 750	0.9991	0.5	1.25	1.25 - 500	0.9980
5α-pregnan-11β,17α-diol-3,20-dione	11β-hydroxy-pdione	11OH-Pdione	348.48	1.25	5.0	5.0 - 750	0.9995	1.25	5.0	5.0 - 500	0.9954
5-pregnen-3β,17α-diol-20-one	17α-hydroxypregnenolone	17OHP5	332.48	5.0	5.0	5.0 - 750	0.9990	5.0	12.5	5.0 - 500	0.9997
5α-pregnan-3α,11β-diol-20-one	3,11diOH-DHP4	3,11diOH-DHP4	334.49	1.25	5.0	5.0 - 500	0.9982	5.0	5.0	5.0 - 500	0.9956
4-pregnen-11β,21-diol-3,20-dione	corticosterone	CORT	346.46	0.05	0.125	0.125 - 500	0.9994	0.125	0.5	0.5 - 500	0.9995
5α-androstan-11β,17β-diol-3,11-dione	11β-hydroxy-dihydrotestosterone	11OHDHT	306.44	0.125	0.5	0.5 - 500	0.9995	0.5	1.25	1.25 - 500	0.9987
4-pregnen-11β,21-diol-3,18,20-trione	aldosterone	ALDO	360.44	0.125	0.5	0.5 - 500	0.9993	0.125	0.5	0.5 - 500	0.9995
5β-pregnan-3α,17,20α-triol	pregnanetriol	Pregnanetriol	336.51	5.0	5.0	5.0 - 1000	0.9967	1.25	5.0	5.0 - 1000	0.9990
4-pregnen-11β,17-diol-3,20-dione	21-deoxycortisol	21dF	346.46	1.25	1.25	1.25 - 1000	0.9991	1.25	5.0	5.0 - 1000	0.9986
4-pregnen-17,21-diol-3,11,20-trione	cortisone	Cortisone	360.45	0.0125	0.05	0.05 - 500	0.9983	0.025	0.05	0.05 - 500	0.9975
5α-androstan-11-one-3α,17β-diol	11-keto-androstanediol	11K-3αAdiol	306.46	0.125	0.5	0.5 - 500	0.9997	0.125	0.5	0.5 - 250	0.9970
5α-pregnan-3α,17α-diol-11,20-dione	11-keto-pdiol	11K-Pdiol	348.48	0.5	1.25	1.25 - 1000	0.9984	0.125	0.5	0.5 - 500	0.9990
4-androsten-11β,17β-diol-3-one	11β-hydroxytestosterone	11OHT	304.42	0.05	0.05	0.05 - 500	0.9989	0.05	0.125	0.125 - 250	0.9956
5α-pregnan-3α,11β,17α-triol-20-one	11β-hydroxy-pdiol	11OH-Pdiol	350.49	12.5	12.5	12.5 - 1000	0.9992	5.0	12.5	12.5 - 500	0.9998

5α-androstan-3α,11β,17β-triol	11β-hydroxy-androstanediol	11OH-3αAdiol	308.46	0.5	1.25	1.25 - 1000	0.9991	5.0	12.5	12.5 - 1000	0.9983
4-pregnene-11β,17α,21-triol-3,20-dione	cortisol	Cortisol	362.46	0.0125	0.05	0.05 - 500	0.9980	0.05	0.125	0.125 - 500	0.9999
4-pregnen-11β,18,21-triol-3,20-dione	18-hydroxycorticosterone	18OH-Cort	362.46	0.125	0.5	0.5 - 1000	0.9993	0.5	1.25	1.25 - 1000	0.9980

* The LOD and LOQ for each steroid were defined as the lowest concentration with a signal to noise ratio (S/N) of >3 for the quantifier ion (LOD), and S/N >10 for the quantifier ion and S/N > 3 for the qualifier (LOQ).

† The calibration curve was selected as the lowest quantifiable concentration to the highest concentration abiding to the linear range. Linearity was determined using Masslynx software.

3.2. Unconjugated free circulating steroid levels in 71 male athletes

We analysed the levels of 57 steroids in the serum of male athletes (Table 2 and Supplementary figure 5.A). Athletes between the age of 18-35 were selected and subdivided into two groups (18-25 [n=40] and 26-35 [n=31] years) and steroid concentrations (Table 2) depicted as the median with IQR as well as the mean with the standard error of mean (SEM).

T was detected in all athletes, falling within the normal range together with the glucocorticoids, cortisol and CORT and their respective inactive forms, cortisone and 11-DHC (Table 2; Figure 4 and 5). While P5 and P4 and their metabolites were present at far lower concentrations than the androgens, as is to be expected, they were also detected in fewer athletes (Table 2; Figure 5). Of interest, however, was 16OHP4, a product of P4 in the Δ^4 pathway, which was detected in 69 athletes. While we have shown that 16OHP4 is metabolised by enzymes in the backdoor pathway [30] no biological function has, to date, been attributed to the hormone. The mean concentrations of the Δ^4 pathway steroid metabolites were far lower than those of the Δ^5 pathway steroid metabolites (Figure 1) together with DHEA being detected in 84 % of the athletes at higher levels than the precursors: P5 < 17OHP5 < DHEA. DHEA was also detected at higher concentrations relative to other C_{19} and C11-oxy C_{19} steroids with the exception of T.

T precursors, A4 and A5 were detected in 70-80 % of athletes but at lower concentrations while the 5α-reduced C_{19} steroids, DHT and 5αDione, and 3αAdiol were detected in fewer athletes — DHT was detected in a third of the athletes with the IQR spanning 0.4986-7.64 whereas 5αDione and 3αAdiol were below the LOQ (Table 2; Figure 4). Although the C_{19} steroid concentrations were higher compared to the C11-oxy C_{19} steroids, the latter steroids were detected in a higher number of athletes with 11OHA4, 11OHT, 11KA4, 11KT and 11K-5αDione detected in 97, 94, 76, 61 and 55 % of the athletes. In addition, the levels of each of the C11-oxy C_{19} steroids, with the exception of 11OHA4 (SEM= 0.209), were detected at similar levels in these athletes (SEM < 0.063). Interestingly, while negligible 11OHDHT (2.0 and 0.3 pmol/L) together with 11K-3αAdiol (1.3 and 2.8 nmol/L) were both detected in only two of the athletes, 3αAdiol (0.06 nmol/L) was detected in one other athlete in whom DHT was below the LOQ (Table 2). Although the C11-oxy C_{19} steroids and their metabolites were all detected with the exception of 11KDHT, 11OH-

5αDione and 11OH-3αAdiol, only four C11-oxy C_{21} steroids were detected in a few athletes 3,11diOH-DHP4, 21dE, 11OH-Pdiol and 11K-Pdiol, with the latter below the LOQ.

The glucocorticoids were present at levels far higher than the androgens (Table 2 and Supplementary figure 5 A) with cortisol and cortisone being the most abundant steroids present in serum, at the highest median concentrations of the steroids measured at 340 and 56 nmol/L respectively. 11-Deoxycortisol, the precursor steroid of cortisol was detected in only 15% of the athletes and at very low levels. The downstream metabolite in the mineralocorticoid pathway, 18OH-CORT was detected in five athletes while high concentrations of ALDO was detected in three of these athletes. Few serum samples contained C11-oxy C_{21} steroids. Of the fifty-seven steroids analysed, twenty-four were not detected and included 11KDHT, AST, 11OHAST, 11KAST, 11OH-5αDione, 11OH-3αAdiol, epiAllo, Pdione, 11αOHP4, 11αOH-DHP4, 11βOHP4, 11βOH-DHP4, 11KP4, 11K-DHP4, Alfaxalone, 21dF, 11OH-Pdione, 11K-Pdione, DOC, epiAST, 3βAdiol, epi11OHAST and epi11KAST.

Table 2: Unconjugated steroids (nmol/L), detected in sera of 71 athletes between the age of 18-35: Data are shown as the number of athletes in which steroid was detected (*No.*), the median with interquartile range (IQR) as well as the mean (±SEM).

Steroid	*No.*	Median (IQR)	Mean (± SEM)
DHEA	60	10.56 (7.322-19.85)	13.65 (1.172)
A5	50	4.249 (2.924-5.99)	4.817 (0.392)
A4	58	1.2 (0.8289- 2.524)	1.883 (0.2216)
T	71	24.28 (20.01-28.99)	24.48 (0.8129)
5αDione	15	0.0006 (0.000473-0.000758) *	0.0006302 (0.00008195) *
DHT	19	1.253 (0.4986-7.64)	4.069 (1.041)
3αAdiol	1	0.05966 (n/a) *	0.05966 (n/a) *
11OHA4	69	3.418 (2.109-4.247)	3.438 (0.2078)
11OHT	67	0.4895 (0.3041-0.6755)	0.5941 (0.06297)
11OHDHT	2	0.00116 (0.000292-0.002028) *	0.00116 (0.000868) *
11KA4	54	0.8129 (0.7151-1.121)	0.8978 (0.04435)
11KT	44	0.3986 (0.2316-0.6739) *	0.5062 (0.06028) *

11K-5αDione	39	0.01263 (0.01135-0.01423) *	0.01318 (0.0004125) *
11K-3αAdiol	2	2.019 (1.281-2.757)	2.019 (0.738)
P5	20	4.898 (3.885-5.565) *	4.793 (0.2697) *
17OHP5	44	7.782 (4.822-12.91)	9.356 (0.9782)
P4	18	0.312 (0.2075-0.6039)	0.3924 (0.05567)
DHP4	1	6.581 (n/a)	6.581 (n/a)
16OHP4	69	0.5266 (0.3204-0.7699)	0.6024 (0.05075)
17OHP4*	39	0.8439* (0.3954-1.928)	1.277 (0.1863)
Pdiol	2	2.358 (1.036-3.679)	2.358 (1.321)
Pregnanetriol	4	3.033 (1.45-17.65)	7.378 (4.992)
3,11diOH-DHP4	1	3.403 (n/a)	3.403 (n/a)
21dE	1	0.09528 (n/a)	0.09528 (n/a)
11OH-Pdiol	2	5.176 (3.51-6.842)	5.176 (1.666)
11K-Pdiol	1	0.000717 (n/a) *	0.000717 (n/a) *
CORT	71	7.713 (4.792-24.84)	13.98 (1.443)
11-DHC	71	4.483 (2.385-5.844)	4.357 (0.2786)
18OH-CORT	5	1.705 (0.8507-29.9)	12.64 (7.119)
ALDO	3	14.42 (1.018-15.15)	10.19 (4.593)
11-deoxycortisol	10	0.4073 (0.09982-1.068)	0.611 (0.2132)
Cortisol	71	339.4 (222.1-465.1)	335.2 (18.2)
Cortisone	71	56.61 (44.49-66.81)	55.4 (2.151)

(n/a) not applicable due to sample number; (*) steroid detected below LOQ

We subsequently compared unconjugated serum steroid levels of athletes grouped according to age (18-25 and 26-35 year). Comparisons of the C_{19} and C11-oxy C_{19} steroids and of the C_{21} and C11-oxy C_{21} steroids showed that there was no statistical difference between the 18-25 and 26-35 age groups for any of the steroids which were quantitated (Figure 4 and 5).

Analysis of the C_{19} and C11-oxy C_{19} steroids (Figure 4) showed that the C_{19} steroid levels were higher (5-fold) than that of the latter group, with 11OHA4 being the most abundant of these steroids detected at far greater concentrations than the other C11-oxy steroids. While 11KDHT was not detected both 11KT and 11OHDHT were lower than DHT. The C11-oxy C_{19} steroid levels

were higher in the two athletes in whom 11K-3αAdiol was detected and while DHT (3 nmol/L) and high levels of T (31 nmol/L) were detected in one of the athletes (age 22), DHT was not detected in the other (age 23) with T levels at 20 nmol/L. Although T and DHT levels were marginally higher in the younger athletes it was not significant and the higher DHT levels were not associated with high T levels, and vice versa.

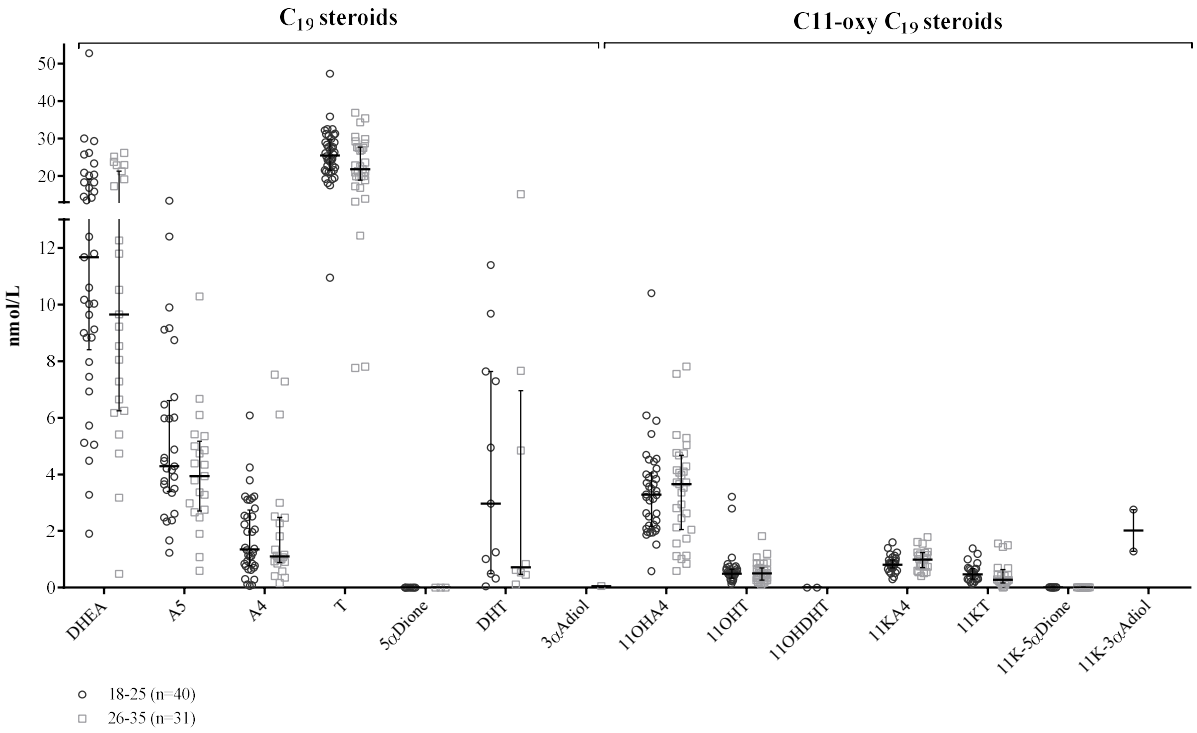

***Figure 4**: Unconjugated circulating C_{19} and C11-oxy C_{19} steroids (nmol/mL). Data of two age groups, 18-25 and 26-35 years, are depicted with bars indicating the median with the interquartile ranges.*

Analysis of the glucocorticoids and mineralocorticoids (Figure 5) show the inactivation by 11βHSD2 with the inactive metabolites (cortisone and 11-DHC) present at significantly lower levels, with the levels not significantly different between the two age groups as in the case of the corresponding active steroid. ALDO was only detected in the younger age group together with their corresponding precursor steroid, 18OH-CORT. While the C_{21} steroids which were detected were present in both age groups, with the exception of DHP4, the steroid concentrations did not differ significantly. Although the C11-oxy C_{21} steroids were only detected in a few athletes, these were only measured in the younger group.

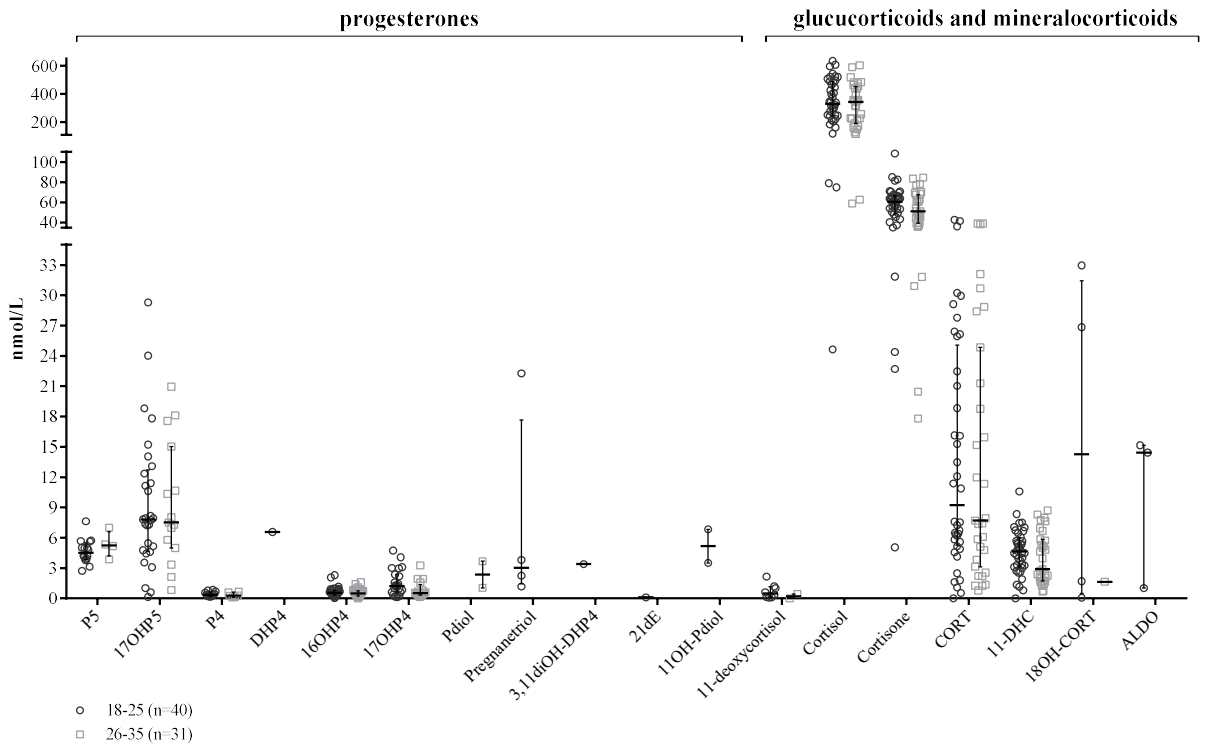

Figure 5: Unconjugated circulating C_{21} and C11-oxy C_{21} steroids (nmol/mL). Data of two age groups, 18-25 and 26-35 years, are depicted with bars indicating median with interquartile ranges.

EpiT concentrations were detected at 129.1 (32.8-424.0) pmol/L for the 18-25 age group and 93.5 (11.8-236.7) pmol/L for the 26-35 age group (Figure 6 A) and showed an average T/epiT ratio of 183 and 210 (Figure 6 B), respectively. No statistical difference was detected between the two age groups for any of epiT. Interestingly, while epiT was detected in 93 % of the subjects, albeit at low concentration, epiAST, 3βAdiol, epi11OHAST and epi11KAST were not detected in any of the athletes. Athletes with high T/epiT ratios had T levels ranging from 13 to 21 nmol/L together with low epiT levels of 0.01 to 0.03 nmol/L while the total steroids measured in these athletes were at the lower end of what was measured in the athletes.

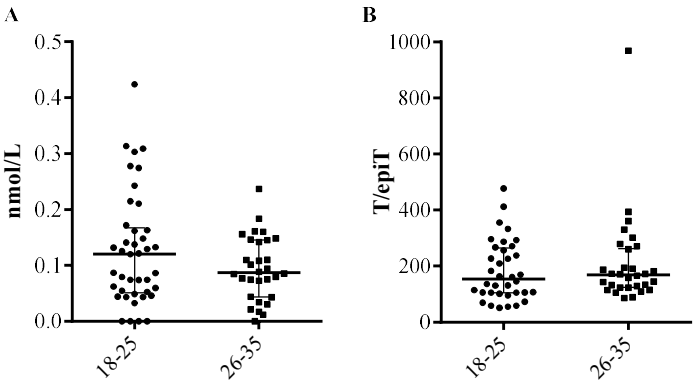

***Figure 6**: Unconjugated epitestosterone (epiT) and testosterone/epitestosterone (T/epiT) ratios in serum. Epitestosterone (epiT) levels (A) and T/epiT ratios (B) in two age groups, 18-25 (n=40) and 26-35 (n=31) years, are represented with bars expressing the median with the interquartile ranges.*

In addition, we compared the steroid levels between the different ethnic groups in serum (Figure 7) and saliva (not shown). No statistical difference was detected between the four groups for any of the detected steroids similar to a report by Litman *et al.* 2006 which showed no difference in T and DHT levels between black, white and Hispanic groups [31]. Other studies have however, reported T levels differing between ethnic groups [32,33]. In our study the sample size was too small to include Asian athletes (2) to confidently conclude on ethnic differences in steroid levels.

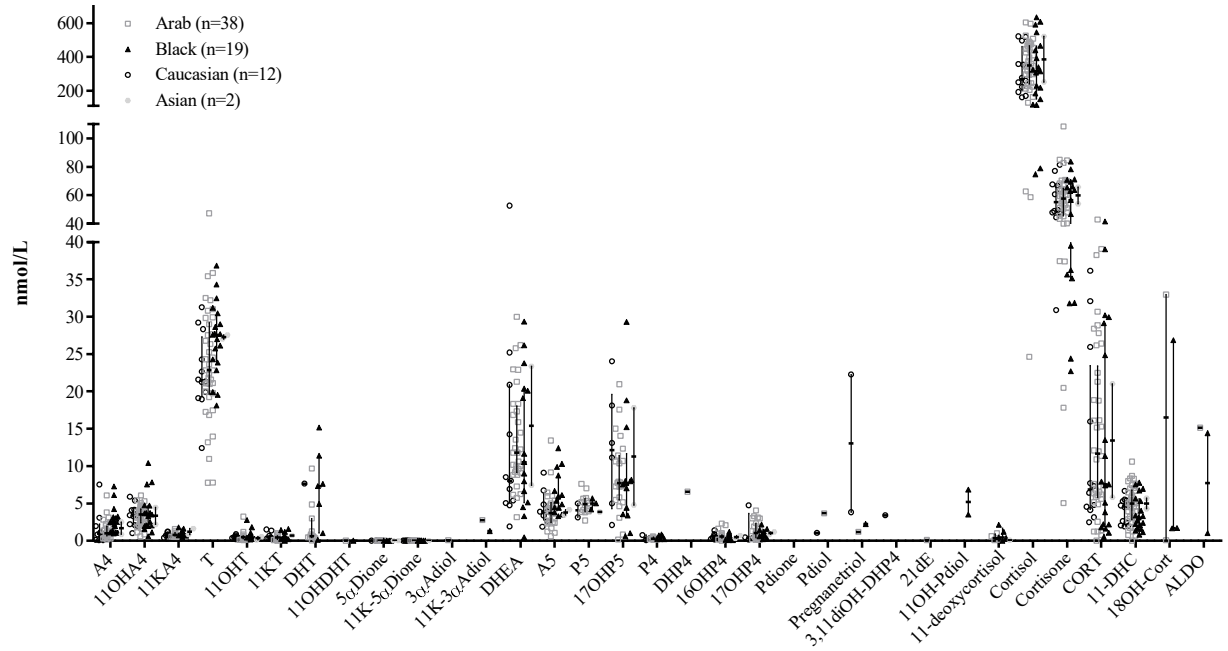

***Figure** 7: Unconjugated circulating steroids (nmol/mL) measured in four ethnic groups, Caucasian, Arab, Black and Asian. Bars indicate median with interquartile ranges. Multiple t test analysis showed no statistical difference in steroid levels between groups.*

3.3. Salivary steroid levels in male athletes

In addition to sampling serum, we also collected saliva from athletes and analysed steroid levels (Figure 8 and Supplementary figure 5 B). Markedly fewer steroids were detected in the salivary samples. Cortisol and cortisone were present in all samples with the latter exceeding cortisol levels significantly. The inactivated derivative of CORT, 11-DHC, was detected in 88% of the samples. The C_{19} and C11-oxy C_{19} steroids were limited to 11KA4 which was detected in 19 samples. P4 and 16OHP4 were also detected, however, present in <15 and < 2% of the athletes, respectively. 11βOHP4 was detected in 48% of the 18-25 age-group and 25% in the 26-35 age-group. No statistical difference was detected between the two age groups with the exception of 11βOHP4 ($p < 0.001$).

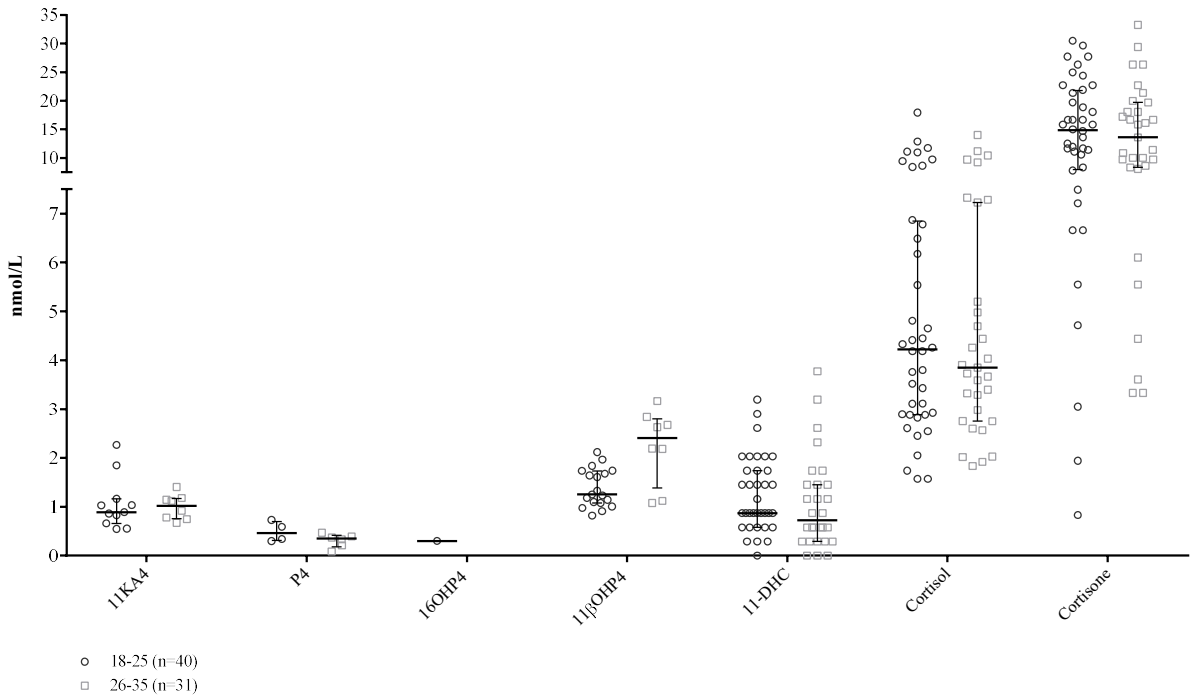

***Figure 8**: Unconjugated C_{19}, C_{21}, C11-oxy C_{19} and C11-oxy C_{21} steroids (nmol/mL) in salivary samples. Data of two age groups, 18-25 and 26-35 years, are represented with bars expressing the median with the interquartile ranges.*

The unconjugated steroid levels detected in both matrices (Table 3) were subsequently compared. The 11KA4 levels were similar at ~1 nmol/L in both serum and saliva while P4 and 11βOHP4 were only detected in saliva. In contrast, cortisol was the major steroid detected in serum with levels significantly lower in saliva (0.016-fold) while salivary cortisone exceeded cortisol levels significantly. Interestingly, while cortisol levels exceeded that of cortisone in serum, salivary levels showed an inverse ratio, with cortisone levels significantly higher compared to cortisol. Similarly, circulating levels of CORT>11-DHC while 11-DHC, only, was detected in saliva.

Table 3: Salivary and corresponding serum steroids, nmol/L, of athletes (age 18-35). Data are expressed as the median with interquartile range (IQR) as well as the mean (±SEM) as well as the saliva: serum fold difference.

	Saliva			Serum		
Steroid	*No.*	Median (IQR)	Mean (± SEM)	*No.*	Median (IQR)	Mean (± SEM)
11KA4	19	0.9169 (0.7484-1.165)	1.035 (0.09915)	18	0.9267 (0.7197-1.156)	0.9697 (0.07661)
P4	10	0.3553 (0.2769-0.4998)	0.3829 (0.05768)	-	-	-
16OHP4	1	0.3026 (n/a)	0.3026 (n/a)	1	0.7623 (n/a)	0.7623 (n/a)
11βOHP4	27	1.607 (1.097-1.967)	1.591 (0.1107)	-	-	-
11-DHC	63	0.871 (0.5806-1.742)	1.18 (0.1081)	63	4.809 (2.72-6.456)	4.713 (0.2804)
Cortisol	71	4.031 (2.883-6.869)	5.236 (0.4023)	71	339.4 (222.1-465.1)	335.2 (18.2)
Cortisone	71	14.7 (8.323-19.98)	14.8 (0.9429)	71	56.61 (44.49-66.81)	55.4 (2.151)

(-) steroids were not detected; below LOD; (n/a) not applicable due to sample number.

We also analysed total steroids (ng) measured per individual in serum and saliva samples (Figure 9 A and B). Steroids in serum ranged between 18 and 315 ng while saliva ranged between 51 and 239 ng with median (IQR) calculated as 170.2 (113.5-221.1) and 135.8 ng (105.1-165.7), respectively. Interestingly, two-way ANOVA analysis with a Sidak's multiple comparison test showed no statistical difference between the total measured steroids of individual serum and saliva.

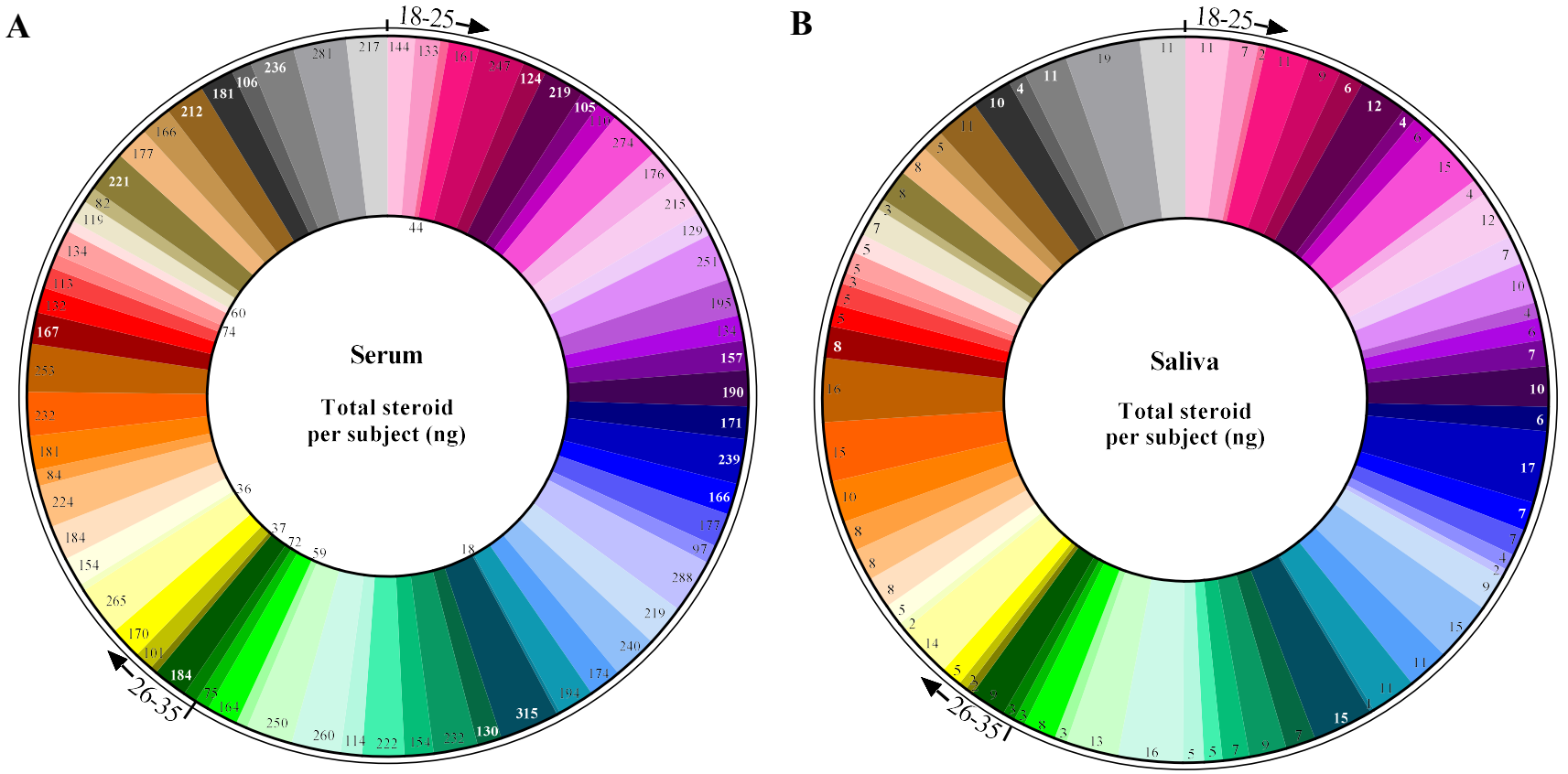

***Figure 9**: Variation in total steroid (ng) measured per athlete. Data of 71 athletes are represented as the sum of all steroids measured in the (A)serum and (B) saliva of the individual athlete between 18-35 years. Outer circle indicates the age group and total ng steroid is depicted on each wedge.*

4. Discussion

Steroid levels are constantly changing throughout the developmental and maturation stages of human life and can span wide ranges, depending on the physiological stage. Studies have reported age-related steroid hormone changes such as during adrenarche, as well as changes brought on by external factors such as stress and lifestyle which include diet and physical activity [34–37]. In this study, we firstly investigated androgen levels together with their epi-derivatives to determine concentrations of unconjugated steroids in circulation in our athlete study group. We also included the analyses of T/epiT ratios even though the aim of this study was not to identify steroid abuse. While the inactive conjugated androgens are generally measured in urinary samples, limited investigations into their circulating levels, and in particular into that of epiT levels, have been reported. It has been suggested that serum could be an alternative to urine for the analysis of epiT levels due to inefficient conjugation resulting from UGT polymorphisms, ethnic differences as well as the use of diuretics and masking agents. The epi-derivatives in our study were unfortunately below the LOD with the exception of epiT which was not detected in five of the athletes with levels ranging from 12-424 pmol/L. EpiT levels have been reported to be age dependent, peaking at ±35 years with levels at ±1.5-2.5 nmol/L [38,39]. While the previous analyses were carried out using RIA and GC-MS, a recent study using ultra-high-performance LC-MS reported epiT levels at 70-400 pmol/L [40]. Distinct differences in urinary glucuronidated steroid levels have been reported for T, epiT, AST, etio and DHEA as well as T/epiT ratios when comparing soccer players within different ethnic groups [41]. While the epiT/T ratios reported were similar in our athlete groups it was only those of Asian descent that differed significantly, however, these were the data of only two athletes.

We detected T as the dominant C_{19} steroid in serum with levels corresponding to reported concentrations ranging from 10-34 nmol/L [34,38,42–44] in addition to A4 and DHEA being similar to reported normal levels with average mean concentration and range ~3.8 nmol/L (1.4-6.2) for A4 [42] and ~20 nmol/L (6.9-40) for DHEA [42,45]. Although epiT is a reported inhibitor of rat CYP17A1 activity [46] *in vitro*, we did not detect increased levels of CYP17A1 substrates (P5 and P4) in those athletes with higher epiT levels.

Since salivary T levels are reported to be ±100 fold lower than T in circulation we were unable to quantify the steroid as levels would be below the LOD. Studies have reported salivary T levels at ~ 250 pmol/L [42,43,45,47,48]. Interestingly, Mezzullo et al. (2017) reported a high linear correlation between salivary

and serum androgen levels including T and A4 with serum T at 20 nmol/L correlating with salivary T at ~300 pmol/L. Given the linear correlation salivary T concentrations of the athletes would be estimated at ~375 pmol/L which is below the LOQ in this study. In contrast, we detected comparable levels of 11KA4 in both serum and saliva, albeit at low levels. Being the only androgenic steroid detected in saliva, 11KA4 therefore could potentially serve as a marker for steroid abuse, easily assessed in saliva. *In vitro* studies have also shown oxidative 17β-hydroxysteroid dehydrogenase (17βHSD) activity in submandibular and parotid glands with A4 being the main metabolite formed in the conversion of T (indicative of oxidative 17βHSD2 activity) together with DHT (indicative of SRD5A activity) [49]. Like that of A4 and T, the interconversion of 11KA4 and 11KT is catalysed by the 17βHSD isoforms — 11KA4 to 11KT by the aldo-keto reductases (such as 17βHSD5 or aldo-keto reductase 1C3 (AKR1C3)) and the reverse reaction by an oxidative isoform (such as 17βHSD2). Interestingly, data show that circulating 11KA4 concentrations in athletes were higher than 11KT concentrations with two athletes having higher 11KT concentrations (1.7- and 2.5-fold) potentially indicating impaired oxidative 17βHSD activity. Neither 11K-3αAdiol nor 11K-5αDione were detected in these athletes further emphasising the importance of measuring downstream metabolites such as the C11-oxy androsterone derivatives, 11K-3αAdiol and 11K-5αDione for clinical and sport related inquiries. Also of note is that in athletes with 11OHA4 levels higher than 5 nM, the adrenal C11-oxy steroid, 11OHT was detected together with 11KA4, and 11KT as well as 11K-5αDione. While studies have been undertaken to investigate salivary steroid levels, these have been limited to mainly cortisol, cortisone, T, DHEA and A4 with the study by Liu *et al.* (2018) being more comprehensive, adding mineralocorticoids, P4 and 17OHP4 [50]. It would be of interest to investigate whether the administration of exogenous 11KT and 11KA4 would impact their circulatory and salivary levels and those of 11K-3αAdiol and 11K-5αDione.

Saliva has become an appealing alternative to serum and urine due to the non-invasive and more dignified collection process with the possibility of multiple systemic sample collection as well as enabling analysis of free unconjugated steroids, potentially reflecting those steroids in circulation [51,52]. Salivary glands, however, also express a number of steroidogenic enzymes which include, amongst others, 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) [53], steroid sulfatase and sulfotransferases (SULT2Bs) and 5α-reductase type 1 (SRD5A1) [53–55]. The expression of 11βHSD2 may account for the higher cortisone levels reported in saliva in comparison to cortisol [45,50]. While cortisol was the major steroid detected in the athlete's serum followed by cortisone which was 6-fold lower, salivary cortisone levels exceeded those of cortisol 3.7-fold. Our salivary cortisol concentrations are comparable

to published concentrations of ~5 nmol/L (1.2-25.8) [45,50,56] while cortisone concentrations fell in the lower range of ~34 nmol/L (11.2-71.9) [45,50]. It is interesting to note that while we did not detect CORT in saliva, 11-DHC was detected in 90% of the salivary samples and together with high cortisone levels, accounting for high cortisone/cortisol ratios, the presence of the high C11-keto steroid levels could be ascribed to 11βHSD2 activity. Despite potential steroid metabolism in salivary glands, it should be noted that steroid passage from circulation to saliva is rapid [49]. The steroid's C11-keto product alfaxalone, catalysed by 11βHSD2, was not detected in saliva while the upstream precursor, 11βOHP4, was detected in ± 40% of the athletes even though the intermediate, 11βOH-DHP4 was not detected. The presence of these steroids indicates that the C11-oxy C_{21} pathway is indeed active *in vivo*. These products furthermore suggest the C11-oxy steroids produced in the adrenal feed into the C11-oxy backdoor pathway as well as into the 11OHA4 pathway.

The role of CYP11B1 is pivotal in the biosynthesis of C11-oxy steroids in the adrenal as is evident in six of the eight steroids detected in saliva with a hydroxyl or a keto moiety at C11. As previously mentioned, 11KA4 was detected at comparable levels in saliva and serum and, could potentially serve as a marker indicating increased CYP11B activity as well modulated 11βHSD and 17βHSD activities. Increased CYP11B1 activity, as in the case of hCG administration resulting in increased 11βOHP4, would also impact levels of 11OHA4, one of the major C11-oxy C_{19} steroids produced in the adrenal. In addition, cortisol, catalysed by CYP11B1 is an indicator of stress and overtraining in athletes. Physical exercise results in an imbalance between cortisol and T levels, however, reports are inconclusive regarding T levels being affected by stress. Stress would influence basal cortisol levels, with T levels (being orders of magnitude lower) being more difficult to interpret. It has also been shown in elite basketball players that match intensity increased both cortisol and T levels, with increased cortisol levels being more significant. T/cortisol ratios are often used to indicate the training load with a decrease in the ratio associated with overtraining, while high ratios are generally recorded in the post-competitive phase [57,58]. Reports do however show that while cortisol and T levels increase with physical exercise, exercise-induced hormone changes are dependent on the intensity of training, type of physical exercise and hydration status of the athlete with steroid levels and T/cortisol ratios returning to basal conditions after 12 h [59,60]. Since CYP11B1 catalyses cortisol production as well as that of 11OHA4, it could be assumed that the latter would also be increased. In our athlete group, the athletes with cortisol levels in the higher range also had 11OHA4 levels in the higher range. Analysis of the athlete's T/cortisol ratios showed ~10-fold lower ratio compared to published free T/cortisol ratio reported between 0.37 and 2.21

[61]. Although circulating cortisol levels corresponded with literature, free T levels were higher [62] which would account for the lower ratio. No C11-oxy C_{19} steroids were detected in the athletes with T/cortisol > 0.4 and while very low cortisol levels were recorded, one of the athletes had high levels of DHT, T and 17OHP4, 7.6, 32 and 2 nmol/L respectively, a very low T/epiT ratio (70) with epiT below the LOD in the second athlete. With steroid levels being influenced by training and competition, it may therefore be prudent to set specific reference ranges which impact not only cortisol but also androgen levels to include the C11-oxy steroids. Although there is no clear evidence that steroid profile ratios are affected by in/out of competition testing, reports suggest that it is commonly perceived to be the case. Measuring the C11-oxy androgens together with T in analyses of complete androgen data sets in anti-doping strategies could perhaps serve to clarify and add to accurate interpretations in terms of steroid analysis.

This study has focussed on analysing a wide range of steroids in athletes other than only the C_{19} steroids generally included in such studies. The fact that we did not detect steroids that have been reported by others in serum and saliva does not detract from the study. Comparisons of steroid levels within a group of participants enables the identification of steroid levels not falling within the norm for the group. These would not necessarily identify doping strategies but could indicate underlying clinical conditions of disorders of the endocrine. One such an example are two athletes with notably high ALDO levels, 15 and 14.5 nmol/L. ALDO production is intricately regulated in the renin-angiotensin-aldosterone system in the maintenance of the electrolyte and water balance in the regulation of blood pressure. The precursor steroids, CORT and 18OH-CORT were also high 11 and 29.9 nmol/L and 33 and 27 nmol/L, respectively. The high ALDO levels could be indicative of an underlying clinical condition or perhaps simply an indication of dehydration. Although these abnormally high ALDO levels may hint at hypertension this would need to be correlated with renin activity. Hypertension aside, these levels may also be indicative of a physiological component such as sodium depletion (or lower intake), dehydration due to intense exercise or medications –illness such as diarrhoea or vomiting would be ruled out as all participants were deemed in good health. Interestingly, these were also the only two athletes in whom 11OHDHT and 11K-3aAdiol were detected while 11OHA4, 11KA4, 11OHT and 11KT were also in the higher concentration ranges.

In conclusion, it is evident that for some steroids the steroid levels vary greatly amongst the athletes tested, together with the levels of total steroids. This highlights the benefit of the biological passport

specific not only to the athlete but also patients in order to monitor and treat endocrine-driven diseases and disorders. Steroid levels in serum and urine have been the topic of numerous investigations with the prerequisite of normal reference ranges being available to enable diagnosis of clinical conditions or to identify steroid doping in the sporting community. The correlation of steroid levels in serum and saliva provides analysis to routinely measure steroid levels such as glucocorticoids. However, known salivary steroid reference ranges are limited to a few steroids and requires further investigations. The advances in investigations into steroid profiles and the development of comprehensive analytical tools and methods enable the detection of novel steroids such as the C11-oxy C_{21} steroid metabolites. In addition, we showed that the steroids containing a C11-oxy moiety are the predominant steroids detected in saliva in this study and provide evidence of C11-oxy C_{19} steroids and C11-oxy C_{21} steroids *in vivo*. Although the physiological role of these C11-oxy steroids remains to be explored, the identification of 11OHA4 pathway metabolites as well as C11-oxy backdoor pathway metabolites *in vivo* clearly underlie the contribution of these pathways to steroidogenic homeostasis.

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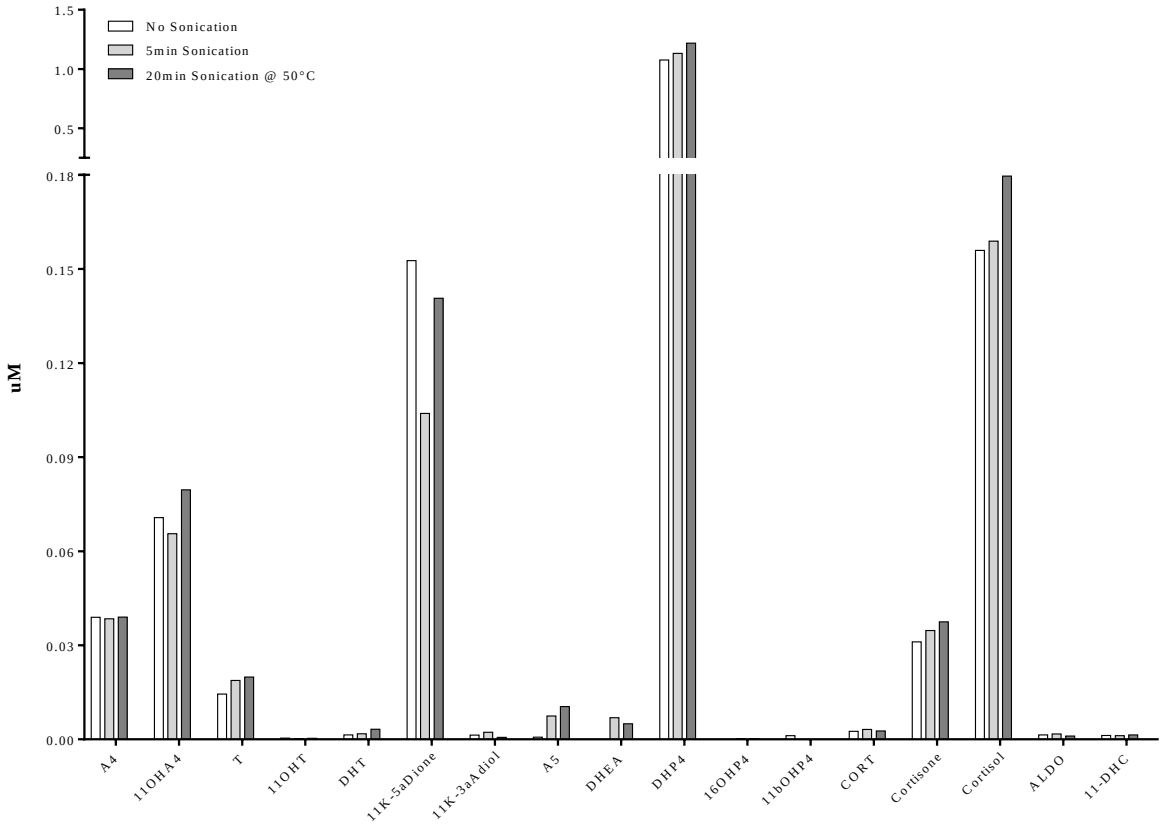
Pre-treatments of serum samples

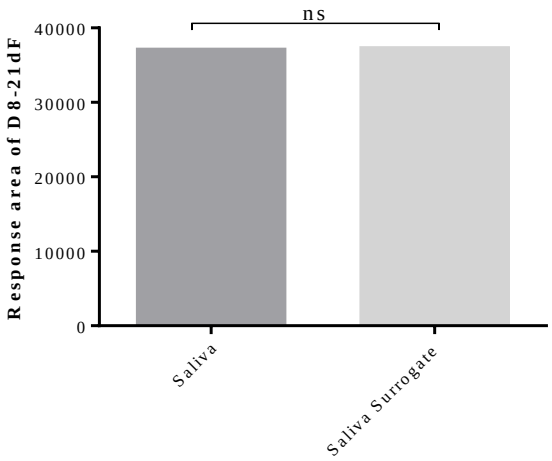
In this study, we compare the free (unconjugated) steroid levels in serum and saliva of elite athletes. Serum, unlike saliva, contains high levels of steroid-binding proteins such as albumin, corticosteroids-binding globulin/transcortin (CBG), sex hormone-binding globulin (SHBG) and progesterone-binding globulin (PBG) which transport the steroids in circulation as dissociable complexes (Burton and Westphal 1972). Studies investigating the steroid levels in serum have reported a sonication step pre-extraction in order to dissociate steroids from steroid-binding proteins. Different protocols were reported: sonication for 5 min at room temperature (Guillette *et al.* 1999) or 20 min at 50 °C (Dorgan *et al.* 2002) as well as extraction without any pre-treatment. We conducted a pilot study to establish the best pre-treatment of serum samples to free protein-bound steroids. Human serum aliquots, 1 mL, were pre-treated, extracted and analysed using an UPC^2-MS/MS method previously published (Du Toit UNDER REVIEW *et al.* n.d.): treatments included no sonication, 5 min sonication at room temperature and 20 min sonication at 50 °C using a Hwashin POWERSONIC 405 sonicator bath. The subsequent extraction with MBTE (Supplementary figure 1) showed that the organic solvent was sufficient in removing bound steroids from binding proteins.

Development of the surrogate saliva matrix

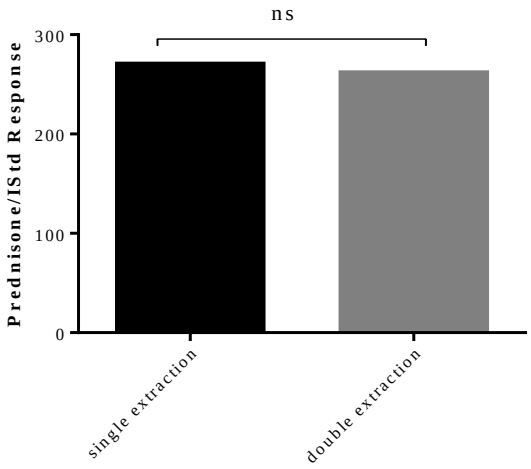
Current studies investigating the steroid levels in saliva use bovine serum albumin (BSA) in PBS as a surrogate matrix. However, saliva composition includes numerous components (proteins, lipids, carbohydrates, electrolytes and enzymes) which may elicit a matrix effect and subsequently influence the accurate determination of steroid levels for which the BSA in PBS matrix would not account for. We therefore prepared a surrogate saliva matrix by dissolving albumen (30%), amylase (25%), lipase (25%) and lysozyme (20%) in phosphate buffer saline (PBS), pH 7, to a final protein concentration of 2 mg/mL, a reported average protein concentration for saliva (Yan *et al.* 2009, Neyraud *et al.* 2011, Kejriwal *et al.* 2014). Proteins were selected based on the molecular weight contribution to the total saliva composition (Yan *et al.* 2009). Aliquots of human saliva and the surrogate matrix were spiked with prednisone and/or deuterated 21dF (D8-21dF), extracted, analysed and neither showed any statistical difference in response (Supplementary figure 2 and 3).



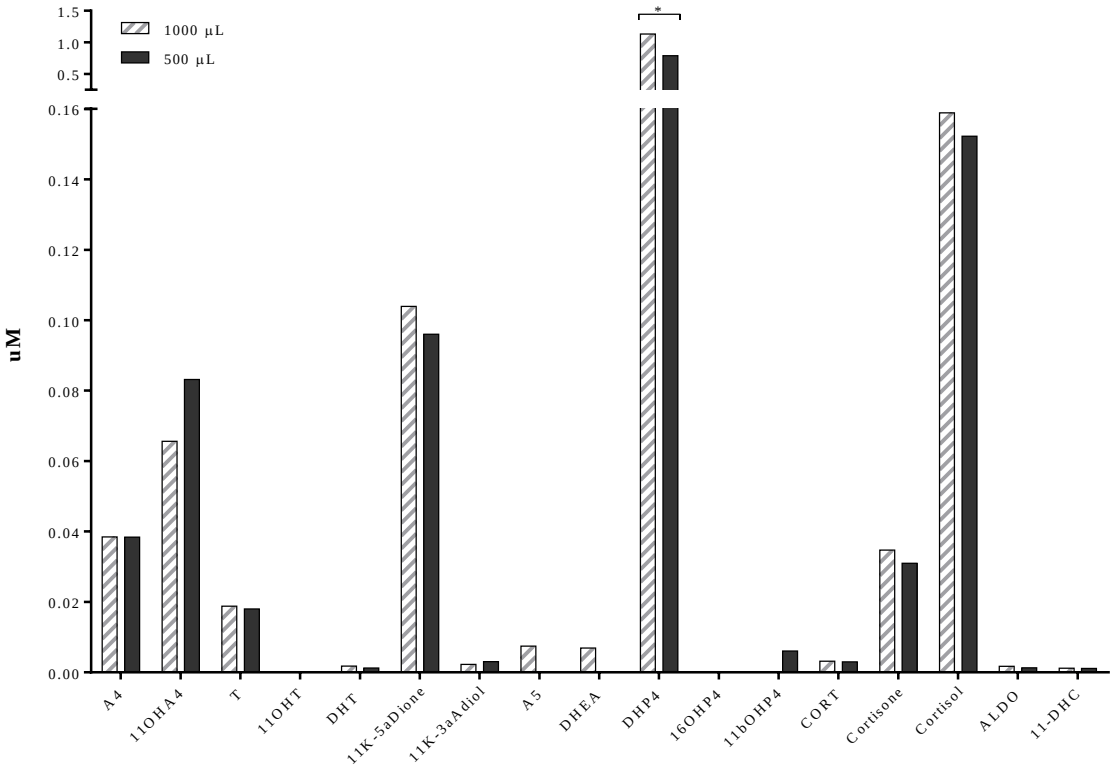
***Supplementary figure 1**: Steroid quantification of 1 mL serum after three pre-treatments; no sonication, 5 min sonication and 20 min sonication at 50 ° C.*



***Supplementary figure 2**: Extraction of 1 ng deuterated 21dF from 1000 µL human saliva and surrogate saliva matrix showed no significant difference in response.*



***Supplementary figure 3**: Single and double extraction of 1ppm prednisone from 1000 µL human saliva and surrogate saliva matrix showed no significant difference in response.*



***Supplementary figure 4**: Steroid quantification, µM, in 1000 µL and 500 µL serum containing internal standard and sonicated for 5 minutes before extraction.*

Supplementary table 1

Elution gradient specifications of the UPC^2 separation of C_{19} and C11-oxy C_{19} steroids

Step	Time (minutes)	Flow rate (mL/min)	Solvent A (%) (CO_2)	Solvent B (%) (methanol)	Gradient curve
1	initial	1.2	96.0	4.0	initial
2	2.4	1.2	95.1	4.9	6
3	3.6	1.2	94.0	6.0	6
4	6	1.2	91.4	8.6	6
5	6.9	1.2	87.0	13.0	6
6	7.2	1.2	75.0	25.0	6
7	7.4	1.2	75.0	25.0	6
8	7.5	1.2	96.0	4.0	6
9	8.5	1.2	96.0	4.0	6

Supplementary table 2

MRM settings of steroid standards used in the UPC^2-MS/MS analysis of the C_{19} and C11-oxy C_{19} steroids. Included were deuterated internal standards and quality control reference standards. Chemical names are depicted with their retention time (RT), molecular weight (Mr), molecular ion species and fragment ion mass transitions, with their respective cone voltages (CV) and collision energies (V).

Compounds		RT	Mr	Mass transitions								
Abbreviation	IUPAC name			Quantifier	Qualifier	Qualifier	CV (V)			CE (V)		
Steroid metabolites												
5αDione	5α-androstan-3,17-dione	1.14	288.4	289.2 > 253.1	289.2 > 271.2	289.2 > 97.2	30	30	30	15	15	22
A4	4-androstene-3,17-dione	1.64	286.4	287.2 > 123	287.2 > 173	287.2 > 211.1	30	30	30	25	20	20
11K-5αDione	5α-androstan-3,11,17-trione	1.77	302.41	303.2 > 241	303.2 > 227.1	303.2 > 248.9	35	35	35	20	20	15
11KA4	4-androsten-3,11,17-trione	2.48	300.4	301.2 > 121.1	301.2 > 265.21	301.2 > 257	35	35	35	30	15	25
AST†	5α-androstan-3α-ol-17-one	2.52	290.4	273.2 > 185.1	273.2 > 69.07	273.2 > 55.14	30	30	30	20	30	30
DHT	5α-androstan-17β-ol-3-one	2.70	290.44	291 > 255.29	291 > 104.9	291 > 159	30	30	30	15	30	25
epi-AST	5α-androstan-3β-ol-17-one	2.87	290.4	273 > 81.11	273 > 134.88	273 > 116.95	15	15	15	30	20	30
11OH-5αDione	5α-androstan-11β-ol-3,17-dione	3.01	290.4	305 > 269.2	305 > 211	305 > 97.2	35	35	35	15	20	30
DHEA	5-androsten-3β-ol-17-one	3.00	288.4	271.2 > 253.2	271.2 > 213	271.2 > 91.05	30	30	30	15	15	40
epi-T	4-androsten-17α-ol-3-one	3.39	288.4	289.2 > 97	289.2 > 175.1	289.2 > 109.25	45	45	45	20	20	25
T	4-androsten-17β-ol-3-one	3.95	288.4	289.2 > 123	289.2 > 271.18	289.2 > 186.8	30	30	30	25	20	20
11KAST†	5α-androstan-3α -ol-11,17-dione	4.00	304.4	287 > 229.1	287 > 147.2	333.1 > 211.25	30	30	30	20	30	20
11OHA4	4-androstene-11β-ol-3,17-dione	4.34	302.4	303.2 > 267.2	303.2 > 285.2	303.2 > 121.1	30	30	30	15	15	25
11KDHT	5α-androstan-17β-ol-3,11-dione	4.75	304.4	305.2 > 243	305.2 > 203	305.2 > 67	30	30	30	20	25	30
epi-11KAST	5α-androstan-3β-ol-11,17-dione	4.65	304.4	287.1 > 228.97	287.1 > 146.99	287.1 > 106.8	30	30	30	20	25	30
3αAdiol	5α-androstan-3α,17β-diol	5.41	292.46	275.2 > 257.25	275.2 > 175	275.2 > 81.1	15	15	15	15	15	30
3βAdiol	5α-androstan-3β,17β-diol	5.63	292.4	257 > 147.06	257 > 161.1	257 > 80.97	40	40	40	20	20	30
11OHAST†	5α-androstan-3α,11β-diol-17-one	5.80	306.44	289 > 271.3	289 > 253.2	289 > 213	15	15	15	15	15	15
A5	5-androsten-3β,17β-diol	5.82	290.4	273.09 > 201.17	273.09 > 185.28	273.09 > 129.14	20	20	20	15	15	30
11KT	4-androsten-17β-ol-3,11-dione	5.95	302.41	303.2 > 147.1	303.2 > 285	303.2 > 107.2	30	30	30	25	15	30
epi-11OHAST	5α-androstan-3β,11β-diol-17-one	6.47	306.4	289 > 151.05	289 > 196.97	289 > 133.13	25	25	25	15	20	20

11OHDHT	5α-androstan-3-one-11β,17β-diol	6.80	306.44	307 > 271	307 > 213.2	307 > 253	25	25	25	15	20	20
11K-3αAdiol	5α-androstan-11-one-3α,17β-diol	7.53	306.46	307.2 > 271	307.2 > 253	307.2 > 159.13	20	20	20	15	20	20
11OHT	4-androsten-11β,17β-diol-3-one	7.74	304.42	305.3 > 269	305.3 > 121	305.3 > 105.1	35	35	35	15	20	30
11OH-3αAdiol	5α-androstan-3α,11β,17β-triol	7.98	308.46	273 > 147.2	273 > 107	273 > 161	25	25	25	20	20	20
Prednisone	1, 4-pregnadien-17, 21-diol-3, 11, 20-trione	7.71	358.43	359 > 147	359 > 171	-	25	25		35	35	
Deuterated steroids												
A4-d7	4-androstene-3,17-dione (2,2,4,6,6,16,16-D7)	1.61	293.45	294.3 > 234	294.3 > 121.15	294.3 > 118.9	25	25	25	20	30	30
AST-d2	5α-androstan-3α-ol-17-one (16,16-D2)	2.51	292.46	275.2 > 215.1	275.2 > 67	275.2 > 54.95	25	25	25	15	30	30
DHT-d4	5α-androstan-17β-ol-3-one (2,2,4,4-D4)	2.68	294.47	295.2 > 163.19	295.2 > 105.05	295.2 > 215.3	30	30	30	20	30	20
DHEA-d6	5-androsten-3β-ol-17-one (2,2,3,4,4,6-D6)	2.98	294.47	277.3 > 219.2	277.3 > 97.2	277.3 > 202.1	25	25	25	15	20	20
T-d2	4-androsten-17β-ol-3-one (1,2-D2)	3.93	290.44	291 > 201.3	291 > 164.84	291 > 105.19	30	30	30	20	20	15
11K-etio-d5	5β-androstan-3α-ol-11,17-dione (9A,12,12,16,16-D5)	4.10	309.4	291.3 > 230.2	291.3 > 273.24	291.3 > 92.97	25	25	25	20	15	30
11OHA4-d7	4-androstene-11β-ol-3,17-dione (2,2,4,6,6,16,16-D7)	4.28	309.4	310.1 > 128.02	310.1 > 168.4	310.1 > 134.95	25	25	25	25	30	30
11KDHT-d3	5α-androstan-17β-ol-3,11-dione (16,16,17A-D3)	4.75	307.4	308.2 > 290.12	308.2 > 131.05	308.2 > 197.15	25	25	25	15	30	20
3αAdiol-d3	5α-androstan-3α,17β-diol (16,16,17-D3)	5.41	295.48	260.2 > 178.1	260.2 > 135.1	260.2 > 109.1	25	25	25	15	15	20
11KT-d3	4-androsten-17β-ol-3,11-dione (16,16,17A-D3)	5.95	305.4	306.2 > 212.95	306.2 > 109.24	306.2 > 171	15	15	15	20	30	30
Quality Control Reference Materials												
(+/-) trans-stilbene oxide		0.64	196.2	197.19 > 105.12	197.19 > 76.99	-	25	25		20	30	
sulfamethoxazole		8.02	253.3	254.06 > 108.06	254.06 > 92.17	-	30	30		25	30	
thymine		4.19	126.1	127.05 > 53.97	127.05 > 55.95	127.05 > 109.97	25	25	25	20	20	20
sulfamethizole		7.62	270.3	271.032 > 92.17	271.032 > 107.99	-	30	30		30	20	

† Note: Although etiocholanolone, 11OH-etiocholanolone and 11K-etiocholanolone were not included in the method, they do crosstalk with AST, 11OHAST and 11KAST, respectively, and may cause a response if present *in vivo*.

Supplementary table 3

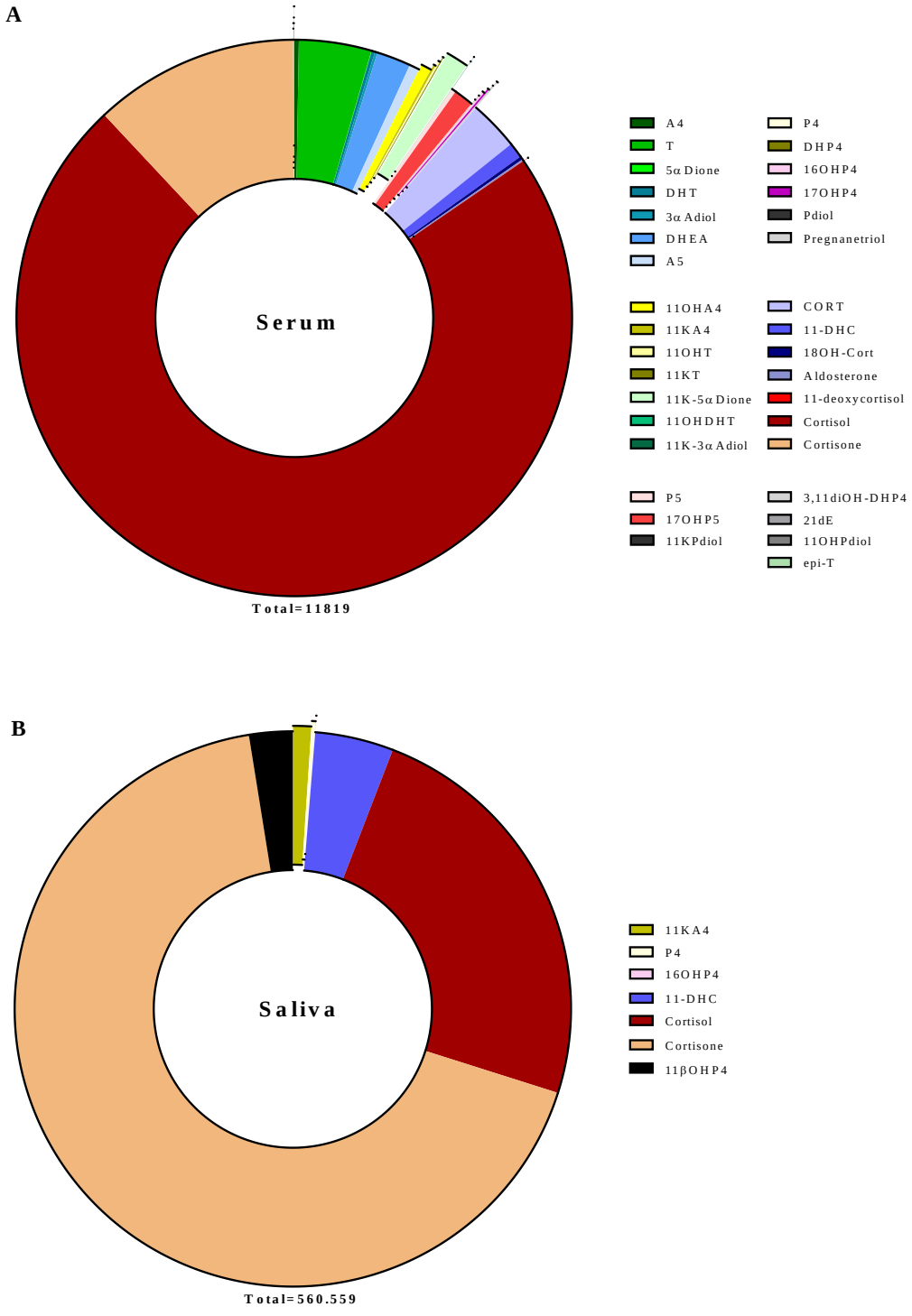
Steroid standards used in the UPC^2-MS/MS analysis of the C_{19} and C11-oxy C_{19} steroids. Method validation was specific to DCC-stripped FBS, 0.5 µL, as a matrix showing calibration range (ng/mL) and linearity (r^2) with the limit of detection (LOD) and limit of quantification (LOQ) shown as ng/mL and pg on column.

Compounds	Mr	RT	LOD*		LOQ*		Calibration	r^2 †
			ng/mL	pg on column**	ng/mL	pg on column**	range†	
Steroid metabolites								
5αDione	288.4	1.14	0.1	0.667	0.5	3.333	0.5 - 1500	0.9991
A4	286.4	1.64	0.1	0.667	0.25	1.667	0.25 – 1250	0.9990
11K-5αDione	302.41	1.77	0.5	3.333	1.0	6.667	1.0 - 2500	0.9941
11KA4	300.4	2.48	0.05	0.333	0.1	0.667	0.1 - 500	0.9995
AST	290.4	2.52	5.0	33.333	10	66.667	10.0 - 500	0.9990
DHT	290.44	2.70	0.1	0.667	0.25	1.667	0.25 - 250	0.9984
epiAST	290.4	2.87	1.0	6.667	2.5	16.667	2.5 - 250	0.9972
11OH-5αDione	304.4	3.01	0.5	3.333	1.0	6.667	1.0 - 1250	0.9999
DHEA	288.4	3.00	0.25	1.667	0.5	3.333	0.5 - 2500	0.9992
epiT	288.4	3.39	0.005	0.033	0.01	0.067	0.01 - 100	0.9988
T	288.4	3.95	0.05	0.333	0.1	0.667	0.1 - 100	0.9967
11KAST	304.4	4.00	0.5	3.333	1.0	6.667	1.0 - 1250	0.9983
11OHA4	302.4	4.34	0.005	0.033	0.01	0.067	0.01 - 500	0.9999
11KDHT	304.4	4.75	1.0	6.667	2.5	16.667	2.5 - 500	0.9988
epi11KAST	304.4	4.65	0.5	3.333	1.0	6.667	1.0 - 2000	0.9994
3αAdiol	292.46	5.41	1.0	6.667	2.5	16.667	2.5 - 2500	0.9997
3βAdiol	292.4	5.63	0.1	0.667	0.25	1.667	0.25 -1000	0.9997
11OHAST	306.44	5.80	2.5	16.667	5.0	33.333	5.0 - 1500	0.9980
A5	290.4	5.82	2.5	16.667	5.0	33.333	5.0 - 500	0.9964
11KT	302.41	5.95	0.1	0.667	0.25	1.667	0.25 - 500	0.9995
epi11OHAST	306.4	6.47	1.0	6.667	2.5	16.667	2.5 - 1500	0.9990
11OHDHT	306.44	6.80	0.1	0.667	0.5	3.333	0.5 - 1500	0.9995
11K-3αAdiol	306.46	7.53	0.1	0.667	0.5	3.333	0.5 - 2500	0.9992
11OHT	304.42	7.74	0.005	0.033	0.01	0.067	0.01 - 500	0.9986
11OH-3αAdiol	308.46	7.98	0.1	0.667	0.25	1.667	0.25 - 2500	0.9993

* The LOD and LOQ for each steroid were defined as the lowest concentration with a signal to noise ratio (S/N) of >3 for the quantifier ion (LOD), and S/N >10 for the quantifier ion and S/N > 3 for the qualifier (LOQ).

** pg steroid in 2 µL injection volume.

† The calibration curve was selected as the lowest quantifiable concentration to the highest concentration abiding to the linear range. Linearity was determined using masslynx software with the lowest r^2 of 0.9941



Supplementary figure 5: *Combined steroid levels (ng) measured in athletes between the age of 18-35. Data of 71 subjects are represented as the sum of the steroid measured in (A) serum and (B) saliva. Steroids are plotted in the order listed from top to bottom, left to right.*

Declaration by the candidate:

With regard to Chapter 7, pages 120-166, the nature and scope of my contribution were as follows:

Nature of contribution: Desmaré van Rooyen completed the following: generated the data reported in the article and all the data in the supplemental file; developed the UPC^2-MS/MS methods (1) pertaining to the epi-derivative analysis and (2) co-developed the method described in Chapter 7; analysed and processed the data; complied all tables and figures, the graphical abstract and the steroid schematic (Figure 1); compiled the first draft of the manuscript, researched articles relevant to manuscript used in the introduction and discussion.

Extent of contribution: data, 100%; manuscript first draft 100%; joint editing of manuscript (50%) with AC Swart.

The following co-authors have contributed to Chapter 7, pages 120-166

Name; e-mail address; nature of contribution; extent of contribution (%)

1. John Molphy: J.Molphy@ljmu.ac.uk; Responsible for sample collection/processing and demographic data. Read and edited the final manuscript draft.
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4. Amanda C. Swart; acswart@sun.ac.za; principal investigator and corresponding senior author.

Signature of candidate: *_____

Date: 9 January 2020

Declaration by co-authors:

The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapter 7, pages 120-166,
2. no other authors contributed to Chapter 7, pages 120-166, besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapter 7, pages 120-166, of this dissertation.

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* Declaration with signature in possession of candidate and supervisor.

Chapter 8

General discussion and conclusion

C11-oxy C_{19} steroids are more frequently detected in hyperandrogenic disorders and androgen-dependent diseases which, to date, mainly include 21OHD, PCOS and PCa with analyses of steroid profiles suggesting 11OHA4 pathway activity. The elucidation of the androgenicity of 11OHT, 11KT and 11KDHT and the prevalence of the C11-oxy C_{19} steroids *in vivo* have directed some focus towards the biological relevance of the C11-oxy C_{19} steroids. In addition, the detection of circulatory 11OHP4 and 21dF in these conditions raises the question as to their biological relevance and whether these steroids should be considered as potential precursors to C11-oxy C_{19} steroids given that backdoor pathway activity has been reported in all of the above-mentioned conditions in which 17OHP4 is converted to AST. The objective of this study was therefore primarily to investigate the biosynthesis of C11-hydroxy and C11-keto C_{21} steroids and to determine whether they are metabolised by the backdoor pathway enzymes subsequently contributing to C11-oxy C_{19} steroid levels. In addition, the steroid levels were evaluated in healthy male athletes to determine the *in vivo* levels which necessitated the development of UPC^2-MS/MS methods to include the novel C11-oxy C_{21} steroids.

Numerous *in vitro* and *in vivo* studies have reported elevated C11-oxy steroid levels such as 11OHA4, 11KA4, 11KT as well as C11-oxy AST derivatives with the latter indicated as a urinary biomarker for 21OHD (Kamrath *et al.* 2017). It is now widely accepted that adrenal 11OHA4 is the 11β-hydroxylated product of A4 with some studies reporting the direct biosynthesis of 11OHA4 from cortisol and 21dF. However, no lyase activity towards these substrates was detected in this study nor was 11OHAST or 11OH-etio biosynthesised from 3α,5α/β-reduced cortisol. Although 11OHAST is generally considered to be a metabolite of 11OHA4, our research group has shown the 3α,5α-reduction of 21dF with the metabolism of 21dF to 11OHAST in LNCaP cells yielding 11OHAST (Barnard *et al.* 2017) which suggests an alternative pathway to 11OHA4 pathway. The presence of elevated C11-oxy C_{19} steroids in clinical conditions suggests functional steroidogenic enzymes which are necessary for C11-oxy C_{19} biosynthesis and include CYP11B1, CYP17A1, 11βHSD2, SRD5A and the HSD enzymes which catalyse the 3α-, and 17β-reductions and oxidations required for androgen production. Therefore, this thesis set out to show that

11OHP4 and 21dF can be metabolised by the full complement of steroidogenic enzymes of the backdoor pathway and can contribute to C11-oxy C_{19} steroids levels associated with hyperandrogenic conditions exhibiting elevated C11-oxy steroid levels and backdoor pathway activity. This study showed the *in vitro* conversion of C11-oxy C_{21} steroids in the backdoor pathway ultimately yielding C11-oxy androgens in the novel C11-oxy backdoor pathway (Figure 8.1).

The results in the present study corroborated with previous results in that CYP11B1 and CYP11B2 catalysed the 11β-hydroxylation of P4 yielding 11OHP4 (Van Rooyen *et al.* 2017). The conversion of 17OHP4 to 21dF by the CYP11B isozymes was comparable (Barnard *et al.* 2017) while P4 was more readily 11β-hydroxylated by CYP11B1 (Chapter 3; figure 3). However, considering the co-expression of CYP11B1 with CYP17A1 in the ZF the production of 11OHP4 would most likely occur in the ZG. Nevertheless, P4 is also produced in the ZF and can thus be converted to 21dF by CYP11B1 and CYP17A1 with either 11OHP4 or 17OHP4 as intermediate. Under normal physiological circumstances the production of 11OHP4 and 21dF would be impeded by the presence of a functional CYP21A2 enzyme that catalyses the production of the CYP11B substrates, DOC and 11-deoxycortisol, which are more readily converted and would subsequently compete with P4 and 17OHP4 for the CYP11B binding site. Extra-adrenal expression of the CYP11B1 have been reported in human testis and ovary (Imamichi *et al.* 2016) as well as in testicular adrenal rest tumours (TART) cells (Lottrup *et al.* 2015, Smeets *et al.* 2015) which would suggest peripheral C11-oxy C_{21} biosynthesis. Furthermore, low CYP21A1 activity and the presence of ACTH receptors in TART cells (Clark *et al.* 1990, Claahsen-van der Grinten *et al.* 2007) would enhance the probability for 11OHP4 and 21dF production. This contribution would be minimal compared to the adrenal. Nonetheless, biosynthesis in tissue such as the testis, where all the enzymes necessary for androgen production are also expressed, may result in C11-oxy steroid production in tissue which would not necessarily reflect circulatory levels. Furthermore, POR deficiency CAH results in diminished CYP17A1 and CYP21A2 activity (Dhir *et al.* 2007) as a consequence of impaired electron transfer suggesting an increased likelihood for the biosynthesis of 11OHP4 as all three adrenal steroidogenic pathways are governed by CYP21A2 and/or CYP17A1. In addition, estrogen production will also be affected, as CYP19A1 activity is also dependent on POR for electron transfer which will prevent the conversion of androgens to estrogens. Interestingly, backdoor pathway activity was increased in POR deficient patients during

fetal-to-early-infantile period while classical androgen biosynthesis was also reported to be impaired (Homma *et al.* 2006).

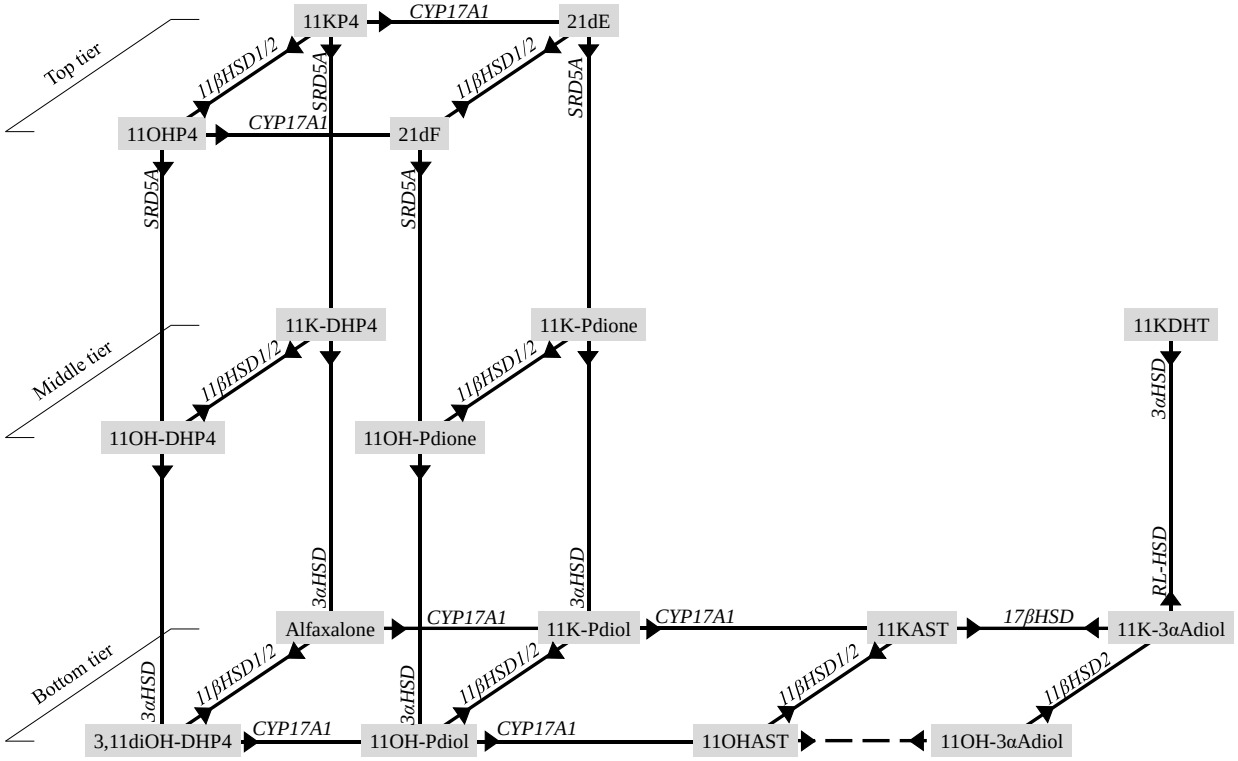

Figure 8. 1 C11-oxy backdoor pathway in which C11-oxy C_{21} steroids are converted to C11-oxy C_{19} steroids.

CYP17A1 can hydroxylate P4 and 11OHP4 to 17OHP4 and 21dF, respectively, but no significant lyase activity was observed which would prevent direct androgen biosynthesis from 21dF in the ZF (Chapter 4, figure 3 A). Therefore, adrenal 11OHP4 and 21dF would be released, albeit at low levels, into circulation where they can potentially elicit a biological effect or serve as substrates for peripheral metabolism by enzymes such as 11βHSD2. This study showed the 11βHSD-catalysed interconversion of 11OHP4 and 11KP4 *in vitro* (Chapter 3 and Figure 5.2) which is supported by Gent *et al.* (2019), who reported a greater catalytic efficiency for the dehydrogenase reaction converting 11OHP4 to 11KP4 compared to the reductase reaction yielding 11OHP4. In addition, 11KP4 was reported as an inhibitor of 11βHSD1 (Gent *et al.* 2019) suggesting that once adrenal 11OHP4 is released into circulation and oxidised by 11βHSD2 it would, unless metabolised further, remain predominantly in its keto form. Higher 11KP4 and 11K-DHP4 levels compared to 11OHP4 was detected in the prostate tissue of BPH patients (Du Toit and Swart

2020). Similarly, a higher conversion of 21dF to 21dE compared to the reverse reaction supports our theory that the metabolism is driven towards the keto moieties. However, this would depend on the tissue expression of the 11βHSD isozyme and cofactor availability and whether the compound is further metabolised. Metabolism of 11OHP4 and 21dF in C4-2B cells established the conversion to their keto derivatives as well as the 5α-reduction of 11KP4 in a cell model system (Figure 5.4). Currently, very few studies include 11KP4 and 21dE in steroids analysis and therefore the physiological levels remain unclear. Previous studies have suggested that 21dF and cortisol are converted to 11OHA4 by the lyase activity of CYP17A1 which contributes to the adrenal 11OHA4 output and urinary C11-oxy C_{19} (Burstein *et al.* 1953, Axelrod *et al.* 1973, Kornel and Saito 1975). However, 11OHA4 was not detected when 21dF and cortisol were assayed in CYP17A1 transfected cells suggesting that 21dF would not be metabolised further in the adrenal (Chapter 4; figure 3 A and 6). Whether the co-expression of CYP17A1 with cyt b_5 may facilitate the cleavage or augment the CYP17A1-catalysed reactions remains to be explored. These findings verify the C11-oxy backdoor pathway to include 11OHP4, 21dF and their keto derivatives which are all substrates for SRD5A and are rapidly converted to their 5α-reduced metabolite by both isoforms (Figure 8.1).

The C11-oxy backdoor pathway further comprises the 5α-reduced steroids which include 11OH-DHP4, 11K-DHP4, 11OH-Pdione and 11K-Pdione. The reduction of the C4-C5 double bond is irreversible thus SRD5A isoforms act as gatekeepers which commit these steroids to the backdoor pathway. The efficient 5α-reduction of 11OHP4 and 11KP4 by either of the SRD5A isozymes, which is more rapid than the 5α-reduction of 21dF and 21dE reported by Barnard *et al.* (2017), introduces these C11-oxy C_{21} steroids into the C11-oxy backdoor pathway, which is a more probable route when compared to the less efficient oxidation of 11OHP4 to 11KP4. The elevated SRD5A1 expression in conditions such as PCa and PCOS further highlights the relevance of considering the C11-oxy C_{21} steroid metabolites in steroid profile analyses with 11K-DHP4 levels detected ~5-fold higher than 11KP4 in BPH tissue (Du Toit and Swart 2020). The higher conversion of 11OH-DHP4 and 11OH-Pdione to their keto derivatives compared to the reverse reaction again suggests that the metabolism is channelled towards the keto moieties (Figure 5.2). However, the reductase and dehydrogenase activity did not differ statistically implying that both reactions are possible (Figure 5.2). The tissue expression and kinetic characteristics of 11βHSD and SRD5A for these steroids would determine the steroid outcome. Furthermore, kinetic studies

would be required to determine whether the C11-oxy C_{21} steroids like 11KP4, are inhibitors of 11βHSD. Interestingly, within the middle tier of the pathway the steroids are restricted to their 11-oxy derivatives or 11,17-oxy derivatives as the 17α-hydroxylase or 17,20-lyase activity towards any of the 5α-reduced C11-oxy C_{21} metabolite were negligible (Chapter 4; figure 3 B). The minimal repositioning of 11OHP4, 11KP4 and their 5α-reduced metabolites in the CYP17A1 active site, in comparison to P4 (Chapter 4; figure 7 A), which is only hydroxylated by CYP17A1, suggests that the C11 moiety and the C4-C5 reduction had negligible effects on the orientation of the steroids. However, while the 17α-hydroxylation of 11OHP4, 11KP4 and DHP4 was similar to that of P4, neither 11OH-DHP4 nor 11K-DHP4 were substrates for CYP17A1 (Chapter 4; figure 3 A and B). Hence, small changes in docking significantly influenced the activity of CYP17A1 towards the 5α-reduced C11-oxy C_{21} steroids. Considering the metabolism of androgens, 5α-reduction yielded more potent androgens which are subsequently inactivated following the 3α-reduction catalysed by HSD enzymes such as 3αHSD3. Unfortunately, this study was unable to conclude on the AR and PR transactivation by the C11-oxy steroids (Chapter 6). Further investigations are required in cell models lacking endogenous steroidogenic nuclear receptors while also monitoring endogenous conversion of the substrate which may provide false data when investigating receptor interaction.

In the backdoor pathway, 3α-reduction of Pdione yielded Pdiol, the final C_{21} steroid metabolite in the backdoor pathway. Investigation into the 3α-reduction of 11OH-DHP4 and 11K-DHP4 showed the production of 3,11diOH-DHP4 and alfaxalone, respectively (Chapter 3; figure 6 and 7). These results and the 3α-reduction of 11OH-Pdione and 11K-Pdione reported by Barnard *et al.* (2017), which was reconfirmed in this study (data not shown), provided evidence that the 5α-reduced C11-oxy C_{21} steroid metabolites are converted by 3αHSD in the backdoor pathway yielding 3α,5α-reduced metabolites —3,11diOH-DHP4, alfaxalone, 11OH-Pdiol and 11K-Pdiol, which forms the bottom tier of the C11-oxy backdoor pathway (Figure 8.1). No statistical difference between the product formation for the 11βHSD-catalysed interconversion between 3,11diOH-DHP4 and alfaxalone was detected (Figure 5.2), suggesting that both steroids can be produced depending on enzyme expression in the target tissue. However, the conversion of 11OH-Pdiol to 11K-Pdiol by 11βHSD2 was higher compared to the reverse reaction also directing the metabolism towards the 11K-derivatives.

Thus far, it is evident that C11-oxy C_{21} steroids are metabolised by the backdoor pathway enzymes yielding 5α- and subsequently 3α,5α-reduced metabolites which form the three tiers of the C11-oxy backdoor pathway (Figure 8.1). The 11βHSD isozymes interconvert the 11-hydroxy and 11-keto derivatives within each tier. Therefore the dominating or specific 11βHSD isoform expressed in the tissue and cofactor ratio will determine which C11 moiety prevails. Furthermore, while CYP17A1 catalysed the hydroxylation of the steroids in the top tier (Chapter 4; figure 3 A) but exhibited no activity towards the middle tier steroids (Chapter 4; figure 3 B) confining these steroids to either the C11-oxy leg or C11,17-oxy leg of the pathway. Analysis of the CYP17A1 conversion of the bottom tier steroids showed the 17α-hydroxylation of 3,11diOH-DHP4 and alfaxalone to 11OH-Pdiol and 11K-Pdiol and the subsequent side-chain cleavage yielding 11OHAST and 11KAST, respectively (Chapter 4; figure 3 C and 4). This study showed the first *in vitro* evidence for the interconversion of 11OHAST and 11KAST as well as the conversion of 11OH-3αAdiol to 11K-3αAdiol. It is therefore evident from this study that for the C11-oxy C_{21} steroids to be converted to C11-oxy C_{19} steroids, the substrate needs to have both the C4-C5 double bond and C3-keto group reduced as none of the top or middle tier steroids yielded C_{19} steroids in the presence of CYP17A1 –nor did the conversion of DHP4 by CYP17A1. Structural docking of 3,11diOH-DHP4 and alfaxalone in the human CYP17A1 active pocket showed a close overlap of the A and B ring compared to 17OHP5 but a greater distance of C17-Fe for 3,11diOH-DHP4 and alfaxalone than 17OHP4 > 17OHP5 which resulted in higher lyase activity (Chapter 4; figure 7 B).

The inability of CYP17A1 to 17α-hydroxylate 11OH-DHP4 and 11K-DHP4 suggests that 11OH-Pdione and 11K-Pdione are specific metabolites of 21dF and 21dE. In addition, the reductase and dehydrogenase activity of HSD enzymes are reversible, thus, suggesting that the bottom tier steroids may possibly be converted back to middle tier steroids resulting in a second alternative pathway to 11OH-Pdione and 11K-Pdione. However, this was not explored in this study. Taken together, the data presented in this thesis clearly shows a novel pathway in which C11-oxy C_{21} steroids are metabolise to C11-oxy C_{19} steroids.

As these steroids have not been described *in vivo*, apart from the precursors, current analytical methods do not include the C11-oxy C_{21} steroid metabolites. To ascertain these steroid levels *in vivo* steroid profiles were analysed in a cohort of athletes. In order to analyse steroid levels in

serum and saliva of male athletes novel UPC^2-MS/MS methods were developed and validated which included a method analysing only C11-oxy C_{19} and C11-oxy C_{21} steroids, a C_{19} and C11-oxy C_{19} method which included epi-steroid derivatives used to analyse the levels of C_{19} and C11-oxy C_{19} steroids in serum as well as a method for the analysis of 52 steroids. These methods were also used to analyse substrate and product levels in enzymatic conversion assays and whole cell steroid metabolism analysis. The latter method characterised steroids of the adrenal steroidogenic pathways, conventional-, alternative- backdoor- and 11OHA4 pathways as well as the C11-oxy C_{21} steroids (Addendum A). The method can be used for routine analysis, providing a high-throughput method which can be applied in future studies to analyse comprehensive steroid profiles of serum and saliva. This method highlights crucial points which are often neglected in current published methods, particularly in complex matrices such as serum, which may result in false positive steroid identification and quantification. For some steroids, sensitivity was sacrificed for accuracy which is crucial for clinical purposes trying to identify active steroid pathways. Sensitivity may potentially be enhanced by alternative extraction methods and the addition of a sample clean-up step, however these are often costly. Ideally one would strive towards a highly sensitive, standardised method which include all known steroids and potential interferences pertaining to a specific matrix which can be used in order to ensure uniformity with regards to steroid profile studies. However, numerous obstacles would need to be overcome for this to realise which include not only the high cost associated with mass spectrometry, but also require advances in computational and mass spectrometry technologies, availability of reference compounds and minimizing the gaps in our understanding of steroid metabolism.

Using this abovementioned method, this study showed the presence of C11-oxy C_{19} and C11-oxy C_{21} steroid levels *in vivo* (Chapter 7). This study quantified only free steroids in serum and saliva and showed higher C11-oxy C_{19} steroid levels in serum compared to the C_{19} steroids. This may, in part be due to the inefficient conjugation of C11-oxy C_{19} steroids. Whether C11-oxy C_{21} steroids are conjugated and/or bind steroid hormone binding protein in circulation is largely unexplored. Further investigations are required to elucidate the bioavailability of these steroids. Although the salivary steroid profile was less comprehensive compared to serum, this study did show the presence of 11KA4 in saliva, suggesting 11OHA4 pathway activity (Chapter 7). Interestingly, all salivary steroids detected contained an oxo-group at C11, except for P4 and 16OHP4, suggesting high CYP11B activity which further highlights the need to investigate the full complement of C11-

oxy steroids. The *in vivo* study also highlighted the variation between individual athletes in terms of their steroid profiles and steroid levels (Chapter 7). Several internal and external factors, ranging from genetic to environmental, influence an individual's steroid profile. In future, the use of comprehensive profiles similar to that of the athletes' biological passport may aid clinical diagnosis, treatments and monitoring of disease and/or disorder progression. It is important to look at the whole picture regardless of whether a steroid is present in low levels or as an inactive metabolite. Steroid precursors, metabolites, different pathways as well as the specific tissue or location provide information which can facilitate in making a more accurate deduction regarding the relevance and/or prevalence of a steroid. This is emphasised by the lower circulating P4 and P5 levels (precursor steroids) compared to cortisol and androgens (end-products) in the athletes' serum, as the biosynthesis of the end-product is dependent on the precursors. If the same is true for the C11-oxy backdoor pathway steroids, one would expect 11OHP4 and 21dF at lower levels relative to the C11-oxy C_{19} steroids. The data nevertheless provides evidence of C11-oxy C_{21} steroid metabolism *in vivo.*

Additional future considerations and recommendations

- Increased 11OHP4 and 21dF levels following ACTH and AngII stimulation due to increased CYP11B expression (Fiet *et al.* 1989, Clyne *et al.* 1997, Costa-Barbosa *et al.* 2010), suggests stimulatory effects of the biosynthesis of C11-oxy C_{21} steroids. Treatments which alter steroidogenesis such as the inhibition of CYP17A1 by abiraterone may also result in increased C11-oxy C_{21} steroid production, particularly in the light of the inhibition of CYP21A1 also having been reported (Malikova *et al.* 2017) ultimately leading to the subsequent metabolism to potent C11-oxy C_{19} steroids. In CRPC, the inhibition of CYP21A2, in addition to CYP17A1 by abiraterone (Malikova *et al.* 2017) would deprive the patient of androgens as well as mineralocorticoids and glucocorticoids which may lead to increased ACTH and AngII levels and the subsequent increase in C11-oxy steroids as the metabolism of P4 by CYP11B becomes more plausible. Although C11-oxy C_{21} steroids would not be converted to androgens by the backdoor pathway due to the inhibition of CYP17A1, 11OHP4 and 21dF can be reduced to the 5α- and 3α,5α-reduced metabolites and exert yet unknown biological effects. The effect of ACTH and AngII should therefore be investigated on the production of C11-oxy C_{21} steroids. The human adrenal carcinoma cell line (H295R), which expresses all the adrenal steroidogenic

enzymes— can be used to analyse the basal and stimulated steroid levels in the presence and absence of abiraterone or CYP21A1 inhibitor to mimic 21OHD conditions.

- Due to the complexity of steroidogenic enzyme expression, their function and regulation, it is possible that the C11-oxy C_{21} steroids are substrates for other HSD enzymes and are metabolised to different degrees in the presence of co-enzymes. It is well documented that the lyase activity of CYP17A1 towards 17OHP5, and to a lesser degree Pdiol, is enhanced by the presence of cyt b_5. However, the influence of cyt b_5 on the conversion of C11-oxy C_{21} steroids remains unknown. In addition, the diverse catalytic activity exhibited by the HSD enzymes suggests that these C11-oxy C_{21} steroids are substrates for the 3β- and 20α/β-activity, an aspect that requires further investigations.
- High circulating levels of 11KP4 has also been detected in neonates (Du Toit *et al.* 2018) which suggests that the C11-oxy C_{21} steroids may be relevant during fetal development. The backdoor pathway activity has been reported to be crucial for normal male differentiation (Flück *et al.* 2011). It would be thus of value to investigate the C11-oxy steroid metabolite levels of the backdoor pathway in the foetus, infants and neonates where the expression profile of backdoor pathway enzymes changes throughout the various developmental stages with the C11-oxy C_{21} steroids potentially being metabolised to C11-oxy C_{19} steroids during normal development.

Taken together, this thesis has shown the *in vitro* metabolism of C11-oxy C_{21} steroids by the backdoor pathway enzymes (Chapter 3 and 4) and has subsequently elucidated the C11-oxy backdoor pathway yielding 5α- and 3α,5α-reduced 11-hydroxy and 11-keto C_{21} metabolites, 11OHAST and 11KAST (Chapter 3, 4 and 5). These findings suggest that the C11-oxy C_{21} steroids can contribute to C11-oxy C_{19} steroid levels and that the C11-oxy C_{21} steroids may be circulating at higher levels as the reduced metabolites —emphasising the importance of considering comprehensive C_{19}, C_{21}, C11-oxy C_{19} and C11-oxy C_{21} steroid profile analyses augmenting our current understanding of the healthy and clinical condition. Although the bioactivity of the C11-oxy C_{21} steroids remain unknown in terms of the PR and AR, the production of 11OHAST and 11KAST which can be reactivated to produce potent androgens indicate indirect physiological relevance. The limited number of studies which do include C11-oxy C_{21} steroids in their investigations are often restricted to measuring circulatory 11OHP4 and/or 21dF which does not necessarily reflect tissue-specific profiles. Furthermore, this study showed the crosstalk between various steroids in UPC^2-MS/MS methods which highlights the importance of considering the

influence of a steroid, regardless of whether it is included in the analysis, on the quantification of another. This study contributes to unravelling the complexities of steroidogenesis and highlights the importance of a comprehensive steroid profile and validated analytical methods to facilitate our understanding of steroidogenesis and its regulation, adaptation and/or manipulation under various conditions such as in diseases and disorders or for anabolic steroid doping purposes.

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Addendum A

Manuscript submitted to Journal of Chromatography B (under review):

Analysis of 52 C_{19} and C_{21} steroids by UPC^2-MS/MS: characterising the C11-oxy steroid metabolome in serum

Manuscript Details

Manuscript number	CHROMB_2020_158
Title	Analysis of 52 C19 and C21 steroids by UPC2-MS/MS: characterising the C11-oxy steroid metabolome in serum
Article type	Full Length Article

Abstract

The C11-oxy androgens have been implicated in the progression of many diseases endocrine-linked disorders, such as polycystic ovarian syndrome (PCOS), congenital adrenal hyperplasia, specifically 21-hydroxylase deficiency (21OHD), castration resistant prostate cancer (CRPC), as well as premature adrenarche. While the C11-oxy C19 steroids have been firmly established in the steroid arena, the C11-oxy C21 steroids are now also of significance. The current study reports on a high-throughput ultra-performance convergence chromatography tandem mass spectrometry (UPC2-MS/MS) method for the separation and quantification of 52 steroids in peripheral serum, which include the C11-oxy C19 and C11-oxy C21 steroids. Fifteen deuterium-labelled steroids were included for absolute quantification, which incorporates steroid extraction efficiency, together with one steroid and four non-steroidal compounds serving as quality controls (QC). The 15 min run-time per sample (16 min injection-to-injection time with a 8 step gradient) quantifies 68 analytes in a 2 µL injection volume. A single chromatographic step simultaneously identifies steroids in the mineralocorticoid, glucocorticoid and androgen pathways in adrenal steroidogenesis, together with steroid metabolites produced in the periphery, presenting an analytical method for the application of screening in vivo clinical samples. This study highlights cross-talk between the C11-oxy steroids, and describes the optimisation of multiple reaction monitoring required to measure steroids accurately. The limit of detection for the steroid metabolites ranged from 0.002 to 20 ng/mL and the limit of quantification from 0.02 to 100 ng/mL. The calibration range for the steroids ranged from 0.002 to 1000 ng/mL and for the QC compounds from 0.075 to 750 ng/mL. The method is fully validated in terms of accuracy (%RSD, <13%), precision (including inter-day variability across a three-day period) (%RSD, <16%), recovery (average 102.42%), matrix effect (ranging from -15.25 to 14.25%) and process efficiency (average 101.79%). The dilution protocol for the steroids, internal standards and QC compounds was validated, while the ion ratios of the steroid metabolites (%RSD, <16%) and QC compounds were monitored and the accuracy bias values (%RSD, <9%) were within acceptable limits. C11-oxy steroid metabolites produced as intermediates in steroidogenic pathways, together with end-products included in the method can potentially characterise the 11β-hydroxyandrostenedione-, C21- and C11-oxy backdoor pathways in vivo. The identification of these C11-oxy C19 and C11-oxy C21 intermediates would allow insight into active pathways, while steroid metabolism could be traced in patients and reference ranges established in both normal and abnormal conditions. Furthermore, conditions currently undefined in terms of the C11-oxy steroids would benefit from the analysis provided by this method, while the C11-oxy steroids could be further explored in PCOS, 21OHD, CRPC and adrenarche.

Keywords	Supercritical fluid chromatography (SFC); Liquid chromatography tandem mass spectrometry (LC-MS/MS); Progesterone (PROG, P4); Polycystic ovarian syndrome (PCOS); 11keto-testosterone (11KT); 11β-hydroxyandrostenedione (11OHA4).
Taxonomy	Supercritical Fluid Chromatography, Steroid, Mass Spectrometry
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Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

Highlights:

- UPC^2 separates 72 analytes in a serum matrix within a 15-min run-time
- Total analytes = 53 steroids, 15 deuterated steroids and four QC compounds
- Unique MRMs differentiate steroids and compensate for steroid cross-talk
- LODs from 0.002 to 20 ng/mL and LOQs from 0.02 to 100 ng/mL
- A targeted steroid metabolome analysis of 32 C11-hydroxyl and C11-keto steroids

Analysis of 52 C_{19} and C_{21} steroids by UPC²-MS/MS: characterising the C11-oxy steroid metabolome in serum

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Abstract

The C11-oxy androgens have been implicated in the progression of many diseases endocrine-linked disorders, such as polycystic ovarian syndrome (PCOS), congenital adrenal hyperplasia, specifically 21-hydroxylase deficiency (21OHD), castration resistant prostate cancer (CRPC), as well as premature adrenarche. While the C11-oxy C_{19} steroids have been firmly established in the steroid arena, the C11-oxy C_{21} steroids are now also of significance. The current study reports on a high-throughput ultra-performance convergence chromatography tandem mass spectrometry (UPC²-MS/MS) method for the separation and quantification of 52 steroids in peripheral serum, which include the C11-oxy C_{19} and C11-oxy C_{21} steroids. Fifteen deuterium-labelled steroids were included for absolute quantification, which incorporates steroid extraction efficiency, together with one steroid and four non-steroidal compounds serving as quality controls (QC). The 15 min run-time per sample (16 min injection-to-injection time with a 8 step gradient) quantifies 68 analytes in a 2 µL injection volume. A single chromatographic step simultaneously identifies steroids in the mineralocorticoid, glucocorticoid and androgen pathways in adrenal steroidogenesis, together with steroid metabolites produced in the periphery, presenting an analytical method for the application of screening *in vivo* clinical samples.

This study highlights cross-talk between the C11-oxy steroids, and describes the optimisation of multiple reaction monitoring required to measure steroids accurately. The limit of detection for the steroid metabolites ranged from 0.002 to 20 ng/mL and the limit of quantification from 0.02 to 100 ng/mL. The calibration range for the steroids ranged from 0.002 to 1000 ng/mL and for the QC compounds from 0.075 to 750 ng/mL. The method is fully validated in terms of accuracy (%RSD, <13%), precision (including inter-day variability across a three-day period) (%RSD, <16%), recovery (average 102.42%), matrix effect (ranging from -15.25 to 14.25%) and process efficiency (average 101.79%). The dilution protocol for the steroids, internal standards and QC compounds was validated, while the ion ratios of the steroid metabolites (%RSD, <16%) and QC compounds were monitored and the accuracy bias values (%RSD, <9%) were within acceptable limits.

C11-oxy steroid metabolites produced as intermediates in steroidogenic pathways, together with end-products included in the method can potentially characterise the 11β-hydroxyandrostenedione-, C_{21}- and C11-oxy backdoor pathways *in vivo*. The identification of these C11-oxy C_{19} and C11-oxy C_{21} intermediates would allow insight into active pathways, while steroid metabolism could be traced in patients and reference ranges established in both normal and abnormal conditions. Furthermore, conditions currently undefined in terms of the C11-oxy steroids would benefit from the analysis provided by this method, while the C11-oxy steroids could be further explored in PCOS, 21OHD, CRPC and adrenarche.

1. Introduction

Liquid chromatography mass spectrometry (LC-MS) applications, as an analytical tool in steroid analysis, has become increasingly popular over the last decade. LC-MS eliminates the drawbacks of cross reactivity, which hampers accurate steroid quantification of immunoassays such as radio-immunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA). Currently, ultra performance LC (UPLC) or ultra-high performance LC (UHPLC) linked mass spectrometry (MS) or tandem mass spectrometry (MS/MS) is routinely operational in the clinical setting to profile patient steroid hormone levels, in addition to quantifying steroids in the research setting [1]. Although gas chromatography mass spectrometry (GC-MS) has long been considered the golden standard of steroid analysis, the technique is highly specialised and laborious with long run-times while also not being as accessible to the researcher as LC-MS. It is being surpassed by an exciting revived chromatographic technique, which combines properties of both GC and LC —supercritical fluid chromatography (SFC) [2]. Already identified during the 1960's and 1970's [3,4], SFC combines high-pressured gas at its critical temperature with the addition of an organic solvent as the mobile phase, as in carbon dioxide (CO_2) modified with methanol. Indeed, this specific combination mobile phase has recently been employed to quantify multiple compounds in different matrices including: gestagens in fat [5]; oxcarbazepine and its chiral metabolites in dog plasma [6]; bile acids and ginesenosides in traditional Chinese medicines [7]; vitamin D metabolites in serum [8]; in addition to the potential of SFC to be applied in metabolomics analysis [9]. Moreover, recent reviews highlight the application of SFC-MS [10], referred to as UHPSFC-MS/MS and as SFC-MS/MS, respectively, in which the application of SFC in *in vivo* steroid profiling is described [11,12].

The advantage of the increased sensitivity, decreased run times and ultimate high-throughput of SFC in ultra-performance convergence chromatography tandem mass spectrometry (UPC^2-MS/MS) [13], provides a platform to not only quantify steroids present at nmolar and pmolar concentrations, but also to characterise steroidogenic pathways. We have fully characterised and reported pathways describing pre-receptor androgens, active androgens and inactive derivatives of the alternative and conventional C_{19} pathways, as well as the 11β-hydroxyandrostenedione (11OHA4)- and the C11-oxy backdoor pathways [14-21]. Both the C_{21} steroids, progesterone (P4) and 17α-hydroxyprogesterone (17OHP4), and C_{19} steroids, androstenedione (A4) and testosterone (T) are substrates for the biosynthesis of C11-oxy steroids. Notably, adrenal cytochrome P450 11β-hydroxylase (CYP11B1) converts A4 and T to 11OHA4 and 11β-hydroxytestosterone (11OHT), respectively [22,23], while peripheral 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) converts these C11-hydroxy steroids to C11-keto steroid hormones, namely 11keto-androstenedione (11KA4) and 11keto-

testosterone (11KT), respectively [23,24]. These four C11-oxy C_{19} steroids, 11OHA4, 11OHT, 11KA4 and 11KT, lay the foundation of the 11OHA4 pathway [25], which has been characterised *in vitro*, with 17β-hydroxysteroid dehydrogenases (17βHSDs), steroid-5α-reductases (SRD5As), 3α-hydroxysteroid dehydrogenases (3αHSDs), uridine diphosphate glucuronosyltransferases, together with 11βHSDs (supplemental Figure 1A) catalysing the biosynthesis of the intermediates [14-16,26]. In this pathway, 11KT and its 5α-reduced product, 11keto-dihydrotestosterone (11KDHT), are androgenic steroid hormones which bind and activate the androgen receptor (AR) [24,25,27-31]. Furthermore, 11OHT and 11β-hydroxydihydrotestosterone (11OHDHT) have also been shown to activate the AR (at 10 nM), comparable to dihydrotestosterone (DHT) [32]. Moreover, 11KT and 11KDHT are conjugated inefficiently both *in vitro* and *in vivo* [14-16], thereby further underscoring their contribution to the active androgen pool. Also contributing to the C11-oxy C_{19} steroid levels are the C11-oxy C_{21} steroid hormones. Cytochrome P450 17β-hydroxylase/17,20-lyase (CYP17A1) converts the Δ5 C_{21} steroids to C_{19} steroids, generally identified by the conversion of 17α-hydroxypregnenolone (17OHP5) to dehydroepiandrosterone (DHEA) in the adrenal and in steroidogenic tissue. Moreover, CYP17A1 also hydroxylates 5α-pregnan-3α-ol-20-one (allopregnanolone) to 5α-pregnan-3α,17α-diol-20-one (Pdiol), with the latter also converted by CYP17A1 to produce androsterone (AST) [33] —suggesting that C_{19} steroid hormones may potentially be biosynthesised from an array of substrates. We recently showed that CYP17A1 also cleaves additional C_{21} steroid substrates, namely 5α-pregnan-3α-ol-11,20-dione (alfaxalone), 5α-pregnan-3α,11β-diol-20-one (3α,11β-diOHDHP4), 5α-pregnan-3α,11β,17α-triol-20-one (11OHPdiol) and 5α-pregnan-3α,17α-diol-11,20-dione (11KPdiol), to produce the C11-oxy C_{19} derivates —11β-hydroxyandrosterone (11OHAST) and 11keto-androsterone (11KAST) [17,18]. Finally, reductive 17βHSDs and oxidative 3αHSDs produce the active androgen, 11KDHT, from 11KAST, thereby substantiating the significance of the C11-oxy backdoor pathway *in vitro* (supplemental Figure 1B and 1C) [17-21]. Importantly, the steroid metabolites in the C11-oxy backdoor pathway have not been described *in vivo*, as current analytical techniques do not include these steroids, or at best only the precursors steroids secreted by the adrenal, such as 11α-hydroxyprogesterone (11αOHP4) [34], 11β-hydroxyprogesterone (11βOHP4) [35] and 21-deoxycortisol (21-dF) [35,36] have been included. Although the full complement of the C11-oxy C_{19} steroid metabolites are not currently included in routine analyses, 11OHA4, 11OHT, 11KA4, 11KT, and 11OHAST and 11KAST, including their 5β-derivatives; 11β-hydroxyetiocholanolone (11OHetiocholanolone) and 11keto-etiocholanolone (11Ketiocholanolone) have been identified as potential markers in clinical conditions marked by androgen excess [31,34,37-39]. The C11-oxy C_{19} steroids have been associated with symptoms in congenital adrenal disorders, specifically 21-hydroxylase (cytochrome P450 21A2) deficiency [39-41], in the development of polycystic ovarian syndrome (PCOS) [42], castration resistant prostate cancer (CRPC) [14,15,43], benign prostatic hyperplasia [16], and premature adrenarche [31]. Given the androgenicity of the C11-oxy C_{19} steroids and their potential as biomarkers of endocrine-related disorders, many research groups are quantifying some of these steroids in serum, plasma, saliva, hair, tissue and urine of patients. However, the contribution of these C11-oxy steroids to disease states will only become apparent once abnormal levels are defined in patient cohorts, also dependent on normal reference ranges. In this regard, recent but very limited progress have been made towards establishing normal reference values [44-46].

The aim of this study was to develop and validate an UPC²-MS/MS method allowing the separation and quantification of the full complement of C_{19}, C11-oxy C_{19}, C_{21} and C11-oxy C_{21} steroid hormones in serum. While we previously reported on a method which quantified 31 C_{19} and C_{21} steroids and their C11-oxy steroid derivatives in serum [47] and in mammalian whiskers [48] using UPC²-MS/MS, these analyses did not include the C11-oxy steroid derivatives —downstream metabolic products of steroidogenic enzymes which include SRD5As, 11βHSDs, 17βHSDs, 3αHSDs and retinol-like hydroxysteroid dehydrogenases (RL-HSDs). In addition to the above, the current method also measures the mineralocorticoid and glucocorticoid hormones produced by the adrenal, as well as androgens produced by steroidogenic tissue. The inclusion of both active and inactive steroid metabolites in our method allows the comprehensive assessment of steroid profiles and corresponding pathways which enable the analysis of not only specific steroid levels and steroid fluxes in pathways, but also the level of inactive steroids which can potentially continue to contribute to the pool of active steroids after inactivation, prior to conjugation and elimination.

2. Materials and Methods

2.1 Materials

Dihydroprogesterone (DHP4), P4, A4, deoxycorticosterone (DOC), DHT, DHEA, pregnenolone (P5), 17OHP4, T, 16α-hydroxyprogesterone (16OHP4), 11-deoxycortisol (11-DOCSOL), 17OHP5, corticosterone, aldosterone, cortisone (E), cortisol (F) and 18-hydroxycorticosterone (18OHcorticosterone) were purchased from Merck (Darmstad, Germany), and 11β-hydroxydihydroprogesterone (11βOHDHP4), 5α-pregnan-11β,17α-diol-3,20-dione (11OHPdione), 3α,11β-diOHDHP4, 11β-hydroxydihydrotestosterone (11OHDHT), 5α-androstan-11-one-3α,17β-diol (11K3αDIOL), 11KPdiol, 11OHPdiol and 5α-androstan-3α,11β,17β-triol (11OH3αDIOL) from IsoSciences (Ambler, PA, USA). All other steroids were purchased from Steraloids Inc. (Newport, RI, USA), with the IUPAC and trivial names and % purity of all steroid metabolites listed in Table 1. Deuterated internal standards were purchased from Cambridge Isotopes Laboratories (Andover, MA, USA), CDN Isotopes (Quebec, Canada) and Cayman Chemical Company (Ann Arbor, Michigan, USA) (Table 1). UPC² quality control (QC) reference material (product#: 186007950-1; lot#: W14051802) was purchased from Waters Corporation (Milford, USA), and included a 1 mL mixture of (+/-) trans-stilbene oxide, thymine, sulfamethoxazole and sulfamethizole (all at 0.5 mg/mL) in acetonitrile/methanol (75/25 v/v). ClinMass®-Optimisation mixes (1 and 2 for steroids) for LC-MS/MS assays in serum/plasma (reference#: MS12015 and MS12016; lot#: 1307) were purchased from RECIPE chemicals and instruments GmbH (Munich, Germany), and included 2 mL mixtures consisting of A4, 11-DOCSOL and 17OHP4 (mix 1; all at 1 ng/µL); and F (5 ng/µL), 21-dF (2 ng/µL), DOC (1 ng/µL) and T (0.5 ng/µL) (mix 2), both in 100% acetonitrile. FOODFRESH CO_2 was purchased from Afrox (Cape Town, South Africa), formic acid and methyl tert-butyl ether (MTBE) from Merck (Darmstad, Germany), methanol 215 SpS from ROMIL Ltd. (Cambridge, England) and fetal bovine serum (FBS) from Biochrom GmbH (Berlin, Germany). The Viridis SFC ethylene-bridged hybrid 2-ethylpyridine (BEH 2-EP) column (130Å, 3.0 × 100 mm, 1.7 µm particle size; part#: 186006582; lot#: 0114381341) fitted with an ACQUITY UPC²® BEH 2-EP VanGuard™

pre-column (2.1 x 5 mm, 1.7 µm particle size; part#: 186006575; lot#: 0109352061) was purchased from Waters Corporation (Milford, USA).

2.2 Preparation of standards

Steroids and internal standards were prepared in absolute ethanol, 1, 2 or 3 mg/mL, depending on availability. Stock solutions of steroids, internal standards and ClinMass®-Optimisation mixes were stored in liquid form at -20˚C, while UPC² QC reference compounds were stored at 4˚C. All solutions were removed from storage only when standard ranges were prepared.

Stock solutions of steroids were used to prepare four master mixes at 20, 200, 1000 and 2000 ng/mL in 50% methanol. These were subsequently used to prepare reference standards ranging from 0.002 to 1000 ng/mL by the addition of the appropriate volume to FBS (matrix). FBS was charcoal stripped of endogenous steroids prior to usage (confirmed by UPC²-MS/MS analysis). Additional samples used for method validation, were prepared by spiking the matrix as well as spiking 50% methanol (no matrix) with the appropriate volumes. Stock solutions of internal standards were used to prepare additional stock solutions in absolute ethanol, 50 ng/uL, for each deuterium-labelled steroid. These solutions were subsequently used to prepare two master mixes, one in 50% methanol and another in deionised water, which was used to spike the reference standards and samples used in the validation of the method. The amount of the internal standards added to the stock solutions were as follows: 1 ng 21-dF-d8, 1.5 ng 11OHA4-d7, 5 ng DHP4-d6, DHT-d4, 11KDHT-d3, 11KT-d3 and F-d4, 10 ng P4-d9, A4-d7, 17OHP4-d8 and T-d2, 15 ng AST-d2, 20 ng 11Ketiocholanolone-d5, and 25 ng DHEA-d6 and 5α-androstan-3α,17β-diol-d3 (3αDIOL-d3). The stock solution of the UPC² QC reference material was used to prepare reference standards ranging from 0.075 to 750 ng/mL in acetonitrile/methanol (75/25 v/v).

2.3 Steroid extraction

Reference standards and method validation samples were prepared in 500 µL FBS, after which 50 µL of the internal standard solution (in 50% methanol) was added to each sample. Liquid-liquid extraction was carried out by the addition of 5 mL MTBE to the samples (1:10), after which the samples were vortexed at 1450 RPM for 15 min. The aqueous phase was frozen at −80°C for 5 min, as previously published [14,20,23,32,47] and the organic phase subsequently collected and dried down under nitrogen at 40°C for 10 min. The dried residue was dissolved in 0.15 mL 50% methanol and the samples stored in LC-MS/MS vials at −20°C, not more than 24 hrs prior to UPC²-MS/MS analysis.

2.4 Separation and quantification of steroid metabolites

Steroids were separated using the UPC²® system (Waters Corporation, Milford, USA), injection volume 2 µL, with an elution gradient (Table 2), in a total run time of 15 min per sample. Solvent A was CO_2 and solvent B methanol. The Viridis SFC BEH 2-EP column was used to separate the steroids, and the column temperature was set to 60°C, while the automated back pressure regulator was set to 1800 psi. A Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, USA) was

used for quantitative mass spectrometric detection and all steroids were analysed in multiple reaction monitoring (MRM) mode, using the positive electrospray ionization mode, with MRM settings shown in Table 3. Preceding the MS line, a make-up pump mixed 1% formic acid in methanol at a constant flow rate of 0.2 mL/min, and the following MS settings were used: capillary voltage, 3.8 kV; ion-source temperature, 120°C; desolvation temperature, 500°C; desolvation gas flow, 1000 L/h; and cone gas flow, 150L/h.

2.5 Method validation

Linear standard curves were generated for each steroid using standards prepared in FBS with the range spanning 0.002, 0.01, 0.02, 0.1, 0.2, 1, 2, 10, 20, 10, 100, 200 and 1000 ng/mL, and extracted as described in Section 2.3. A blank matrix (matrix only, no internal standard) and a zero standard (matrix with the addition of internal standards) were also included in the standard curves. The limit of detection (LOD) for each steroid was defined as the lowest concentration at which a signal-to-noise (S/N) ratio measured for the quantifier ion (QN) was > 3. The limit of quantification (LOQ) was defined as the lowest concentration for each steroid at which a S/N ratio >10 for the QN and a S/N ratio >3 for the qualifier ion(s) (QL) was measured. The LOQ of each steroid was also confirmed with ion ratios, <15% relative standard deviation (RSD). Linearity of the standard curves was assessed and confirmed using the Runs test in Graphpad Prism (version 8).

In the current study, accuracy is defined as the %RSD from the average response following repeated injections of a single sample and precision is defined as the %RSD from the average response following the injection of independent replicate samples, with the latter repeated over three days to assess inter-day variability [47]. Accuracy and precision were determined for the following reference standard concentrations: 2, 20, 100 and 200 ng/mL by the addition of the appropriate volumes to FBS (matrix) prior to extraction. Recovery, matrix effects and process efficiency were determined at the same concentrations, in parallel with accuracy and precision, according to published protocols [49]. Recovery was calculated by comparing the response of the steroid standards prepared in FBS and extracted as described above, to that of FBS extracts with the post-addition of the same volume (and concentration) of steroids. Matrix effects were determined by comparing FBS extracts with the post-extraction addition of steroids, to a pure solution in 50% methanol (no matrix; no extraction) to which the same volume (and concentration) of steroids were added. The difference in response between these two samples was divided by the response of the pure solution in order to calculate the matrix effect. The process efficiency was calculated by comparing the response of standards prepared in FBS (pre-extraction addition of steroids) to those prepared in 50% methanol. Carry over was determined by quantifying the steroid concentration in blank matrix samples run after the 200 and 1000 ng/mL calibrators and the resulting response values were compared to generate percentage carry over values. In addition, the standard range was run from low to high concentrations, followed by running the same standard range from high to low concentrations [50], and comparing the response values and resulting gradients from both runs. The dilution protocol was validated by diluting the 200 ng/mL precision samples 10-fold with the serum matrix and comparing the average response values with the average response values determined for the 200 ng/mL precision samples (% recovery). Additionally, %RSD values were also determined for the diluted samples.

The determination of response factors (Rf) required the preparation of reference standards ranging from 2 to 1000 ng/mL, which was extracted from 500 µL serum matrix (according to section 2.3). The steroid combinations for comparison were as follows: combination 1, 11αOHP4, 11keto-progesterone (11KP4) and corticosterone, which was compared to combination 2, P4 and F; combination 3, A4 and DHP4, which was compared to combination 4, T and Pdione; and combination 5, 3α,11β-diOHDHP4, 11βOHDHP4, 11KT, 11OHPdiol, 11OH3αDIOL, 11KAST, which was compared to combination 6, 11βOHP4, 11OHT, 11KA4, 11KPdiol and 11K3αDIOL. These combinations were prepared as stock solutions at 20, 100, 200 and 1000 ng/mL in 50% methanol and stored at -20˚C until use. The determination of accuracy bias required two standard mixes to be prepared, in-house, which match the ClinMass®-Optimisation mixes (in acetonitrile). These mixes, together with the ClinMass®-Optimisation mixes were used to prepare reference standards ranging from 0.2 to 1000 ng/mL for comparison. These reference standards were prepared in FBS (500 µL) and extracted according to section 2.3, after the addition of 50 µL internal standard mix in 50% methanol. Acetonitrile alone was extracted and analysed as a matrix control. Accuracy and precision of the internal standards were determined by spiking the serum matrix (500 µL) with 50 µL of the internal standard solution (in 50% methanol) and extracting these samples as described above. The dilution protocol was further validated by diluting the internal standard precision samples 3-fold and 10-fold with the serum matrix. The resulting peak areas were integrated and the %RSD determined. The UPC^2 QC reference material was also validated, and accuracy and precision (plus inter-day variability) were determined at 3.75, 37.5, 75 and 375 ng/mL. The dilution protocol was also validated by diluting the 375 ng/mL accuracy and precision QC samples 10-fold with the serum matrix, and the precision samples were assessed over three days. The resulting peak areas were compared with the 37.5 ng/mL precision QC samples and %RSD values were calculated for both accuracy and precision. In addition, acetonitrile/methanol (75/25 v/v) alone was extracted and analysed as a matrix control for these samples.

2.6 Data processing

Data collection and processing was completed using Masslynx 4.1 software program (Waters Corporation). Steroids were quantified relative to their QN and the QN of the appropriate deuterium-labelled internal standard, which was chosen based on retention time (RT). A smoothing setting of 1/2 was applied to all peak integrations and peak area was used for quantification. The internal standard concentration was fixed to 1, and Targetlynx produced standard curves, weighted 1/x, excluding the origin, such that the line was not forced through zero.

3. Results

3.1 UPC^2 separation of steroid metabolites, internal standards and QC compounds

The analytical method described in this study included 52 steroid hormones (and prednisone as an internal control), 15 deuterium-labelled steroids and four QC compounds (Table 1). This required the optimal chromatographic separation of the steroids and compounds which would permit the accurate quantification of steroids using their identification through mass determination of ion species (Table 3). MRMs were thus determined for each steroid and included the selection of specific molecular

ions, together with cone voltages and collision energies at which these ions are generated. This posed challenges with steroids being structurally similar, together with the C_{19} and C_{21} steroids included in our method having an average molecular weight of 323.64±22.81, as well as 13 steroid groups, including groups of two, three, four, five and six steroids, having similar molecular weights (Table 1). It may be that steroids elute at the same RT, but do not cross-talk, but said steroids may nevertheless cross-talk in the channels (or MRMs) of other steroids —this cross-talk can in turn be either at the same RT or at a different RT. The MRMs circumvented and addressed the problem of differentiating steroids of similar structure and molecular weight, steroid cross-talk and those that could not be separated chromatographipally on the optimised gradient (Table 2). The QN for the specific steroid was chosen based on zero to minimal cross-talk (with a S/N ratio of the cross-talk peak <10), and the ion ratios (QL/QN) were determined not to be influenced (%RSD <15%; supplemental Table 1). Data depicting cross-talk is shown graphically in Figure 1, which highlights that steroid hormones have the tendency to cross-talk and that the gradient and run time of an analytical method must be optimised in order to allow for optimal separation. Moreover, using UPC^2-MS/MS we were able to identify steroid hormones that were contaminated with their precursor steroids used during chemical synthesis (% purity reported in Table 1), with information regarding contaminants and method of synthesis not generally made available by suppliers. Steroids contaminated with traces of their precursor steroids (in curly brackets) were as follows: Pdione {DHP4}, 11αOHP4 {P4}, 11KP4 {P4}, 11KT {11KA4 and 11OHT}, A4 {T}, 11βOHDHP4 {11βOHP4}, 11OH3αDIOL {11K3αDIOL}, 11OHPdiol {11KPdiol} and corticosterone {F}. These steroids required the calculation of Rf values to allow for their absolute quantification, which was determined in a serum matrix (supplemental Table 2). The same applied to A4, 5α-androstan-3,11,17-trione (11K5αDIONE) and 11keto-dihydroprogesterone (11KDHP4), as their MRMs were also optimised for cross-talk from the serum matrix. A peak was observed eluting between 1.8 and 2.15 min, spanning the RTs of A4, 11K5αDIONE and 11KDHP4, which complicated peak integration. Unique MRMs were therefore determined for these three steroids for accurate quantification in serum.

The constraints placed on the successful quantification of the number of steroids included in our analysis by steroid cross-talk was most apparent for the steroid pairs P5 and 5α-pregnan-3β-ol-20-one (epiallopregnanolone), with the only difference being the C5-C6 double bond, and AST and Pdione, with the keto and hydroxyl moieties being on either C3 or C17 and the acetaldehyde side chain of Pdione at C17 (Table 1). More than 40 fragment ions were considered for each steroid in order to compensate for the cross-talk and allow for accurate quantification. It should be noted that fragment ions that include common losses (such as the loss of a water molecule) were avoided as these can be non-specific for the steroid analytes. However, for certain steroids, taking into account the loss of one or two water molecules as the molecular ion specie in the MRM, increased ionisation of the steroid and in some cases decreased cross-talk signals. These steroids were therefore quantified using these molecular ion species, instead of $[M+H]^+$. The steroids for which $[M-H2O+H]^+$ was used included AST, 3αDIOL, 11OHAST, 11KAST, 3α,11β-diOHDHP4, epiallopregnanolone, Pdiol, 11OHPdiol, 11KPdiol, P5, DHEA and androstenediol (A5) and for which $[M-2H2O+H]^+$ was used included 11OH3αDIOL, 17OHP5 and pregnanetriol.

The high-throughput UPC^2-MS/MS method was accomplished with the chromatographic separation and quantification of 53 steroid metabolites in a single 15-min run-time (Figure 2) —the run time

spanning injection-to-injection being 16 min with the inclusion of the sample acquisition to the next sample acquisition. Data was collected up until 13.7 min, followed by a column wash for 1.3 min with the same solvent. After each run the delta pressure was returned to 15 psi and the system pressure started and returned to 2985 psi per run (supplemental Figure 2). Each chromatographic peak was defined by 15.27 points per peak (accepted minimum, 10 points per peak). The window of steroid detection was chosen based on the RT and peak width, to which an additional 0.5 min was added at the beginning and at the end to ensure optimal resolution. The deuterium-labelled steroids eluted marginally earlier than their equivalent analytes, with RT differences ranging from 0.01 to 0.12 min (average, 0.44 min), while only 11Ketiocholanolone-d5 (5 deuteriums and a C3 hydrogen in the α-configuration) (Table 1) eluted 0.24 min after 11KAST.

Internal standards are required to be labelled with deuterium at 3 or more positions in order to be the most appropriate for absolute quantification. The labelled steroids used as internal standards contained 2 - 9 deuterium atoms (Table 1) with their elution profiles shown in supplemental Figure 3. Although T was labelled with deuterium at C1 and C2 and AST was labelled at C16 in the α and β configuration, these two internal standards did not contribute to peaks in their non-deuterium labelled steroid channels. The MRMs of T-d2 and AST-d2 were optimised so that no cross-talk was observed in their channels when T and AST were present, nor in the channels of T-d2 and AST-d2 when T and AST were present. In this regard, none of the internal standards included in this method, even when present at high concentrations (2 ppm), elicited cross-talk with their natural isotopes and *vice versa*, regardless of impurities which may have been present (Table 1).

Although the following steroids eluted closely or at the same RT, no cross-talk was detected: 5α-androstan-3,17-dione (5αDIONE) and DHP4; P4 and A4; 11K5αDIONE and 11KDHP4; 11KA4 and AST; DHEA, epiallopregnanolone and 5α-androstan-11β-ol-3,17-dione (11OH5αDIONE); 11OHA4 and 5α-pregnan-17α-diol-3,11,20-trione (11KPdione); 3αDIOL and 21-deoxycortisone (21-dE); and aldosterone and pregnanetriol. It should be noted that in chromatographic separations in methods using similar settings and column chemistry, the following steroids should be separated by the elution gradient as their ionic species may be present in the same channels: A4 and 11KDHP4; 11KDHP4 and DOC; AST and DHT; 5α-pregnan-11α-ol-3,20-dione (11αOHDHP4) and alfaxalone; 11βOHP4, 16OHP4 and 11αOHP4; 11-dehydrocorticosterone (11-DHC) and 21-dE; aldosterone and E; 21-dF and 11KPdiol; and F and 18OHcorticosterone (supplemental Figure 4).

The synthetic glucocorticoid, prednisone, was added as an internal control to this method. This steroid should not be present in serum, and serum samples could therefore be spiked with prednisone to determine the extraction efficiency of samples. However, prednisone elutes closely to 11OHT and while 11OHT did not cross-talk with the MRMs used to identify prednisone, the 11OHT peak may be overshadowed by the presence of prednisone at high concentrations; therefore prednisone's concentration was ≤2 ng/mL as to not influence the peak resolution of 11OHT. The QC compounds which eluted in the beginning, middle and at the end of our elution gradient (Figure 3), served to assure that the method was fully operational and working correctly, and the ion ratios and RTs of (+/-) trans-stilbene oxide, thymine, sulfamethozaxole and sulfamethizole were thus reviewed during the run.

3.2 Performance and validation of the method

3.2.1 Calibration range of the steroid metabolites and QC compounds

The standard curves for the analytes included minimum 4-points (for Pdione) to 14-points (for F) linear calibration curves with acceptable linearity determined for all curves represented by r^2-values ranging from 0.9974 to 0.9999 (average 0.9996). In addition, the calibration curve for each steroid was confirmed by acceptable %RSDs for the ion ratios (supplemental Table 1). The LODs ranged from 0.002 to 20 ng/mL, while the LOQs ranged from 0.02 to 100 ng/mL (Table 4). The upper limit of quantification (ULOQ) could only be determined for prednisone, >1000 ng/mL (>6666.67 pg on the column) and A4, ≥ 1000 ng/mL (≥ 6666.67 pg on the column). The standard curves for the QC compounds included 9-point linear calibration curves with their linearity (r^2) ranging from 0.9972 to 0.9997, the LODs from <0.075 to 3.75 ng/mL, and the LOQs from 0.075 to 7.5 ng/mL (Table 4).

3.2.2 Carry over

The following steroids showed carry over: corticosterone, 0.36%; aldosterone, 1.09%; E, 0.48%; 11OHT, 0.12%; F, 1.71% and 18OHcorticosterone, 1.68%. It should be noted that the 200 and 1000 ng/mL calibrators used to measure carry over allowed 400 and 2000 pg of the steroids on the column, respectively. The carry over by 18OHcorticosterone (RT, 13.28 min; LOD, 0.2 ng/mL) only amounted to 2.89 nM carry over following the injection of the 200 ng/mL calibrator and 14.62 nM following the 1000 ng/mL calibrator (%RSD gradient difference, 0.46) and F (RT, 13.15 min; LOD, 0.002 ng/mL) showed carry over following the injection of the 200 and 1000 ng/mL calibrators, 2.99 and 14.06 nM, respectively (%RSD gradient difference, 1.80). Aldosterone (RT, 11.65 min; LOD 1 ng/mL) showed carry over following the injection of the 200 and 1000 ng/mL calibrators, 1.89 and 8.65 nM, respectively (%RSD gradient difference, 4.32). It should be noted that in order to limit any possible carry over, blank samples of 100% methanol were injected following the injection of high calibrators throughout the validation of the method.

3.2.3 Accuracy and precision of the steroid metabolites, internal standards and QC compounds

The accuracy and precision, including the dilution protocol, were determined at at least three concentrations for each steroid and QC compound, with most steroids, sulfamethoxazole and sulfamethizole being tested at four concentrations, Pdione assessed at two concentrations and the internal standards tested at one concentration.

3.2.3.1 Accuracy and precision

Acceptable %RSDs (<13%) were obtained for the steroid metabolites, internal standards and QC compounds for the accuracy parameter (Table 5). Accuracy %RSD averages for the steroids ranged from 4.93% at the lowest concentration tested, to 2.15% at the highest concentration tested (overall average %RSD, 3.06%; n=191). The best accuracy was achieved for both 11KAST and 11OHDHT at 200 ng/mL, 0.45%, while the accuracy for 11KDHP4 and 21-dF was 12.77% at the lowest

concentration tested. The accuracy %RSD for DHP4-d6 (11.52%), DHT-d4 (11.74%) and 11KDHT-d3 (10.61%) were >10%, while the %RSDs for all other internal standards were <10%. The accuracy %RSD averages for the QC compounds were 5.90% for (+/-)-trans-stilbene oxide, 5.33% for thymine, 2.70% for sulfamethoxazole and 5.64% for sulfamethizole —overall the %RSD values ranged from 2.04% to 8.56%.

Precision for the steroid metabolites, including inter-day variability, produced %RSD values ranging from 0.28% (for 11KA4 at 2 ng/mL on day 2) to 15.32% (for 11OHPdione at 20 ng/mL on day 1), with the overall average %RSD being 6.94% on day 1, 3.31% on day 2 and 5.56% on day 3, across the four concentrations tested each day (Table 5). Precision was determined to be <10% for all steroids, on all three days, at 100 and 200 ng/mL, except for 11OH3αDIOL (13.63%), 11αOHDHP4 (10.68%), alfaxalone (10.99%) and 11OHPdione (10.29%) testing >10% at 100 ng/mL and 11-DHC (11.69%), Pdiol (10.97%), 11OHPdione (10.41%) and 11αOHDHP4 (11.16%) testing >10% at 200 ng/mL. The precision %RSD average was 6.02% for the deuterated steroids and tested <10% for all the internal standards, except for DHP4-d6 (10.07%) and AST-d2 (10.34%). The precision parameter tested <11% for all the QC compounds across the three-day period at all four concentrations tested, except for sulfamethizole at 3.75 ng/mL, being 16.47% on day 1 and 15.75% on day 2.

3.2.3.2 Accuracy and precision of the dilutions

Validation of the dilution protocol was determined for the steroid metabolites, internal standards and QC compounds, shown in supplemental Table 3, 4 and 5, respectively. The precision %RSD values for the 10-fold dilution of the steroid metabolites were <15%, ranging from 2.81% for Pdione to 14.80% for 17OHP4, while the percentage recovery average was 93.27% (n=53) (supplemental Table 3). A 3-fold dilution of the internal standards measured precision %RSD values ranging from 1.83% (for 11KDHT-d3) to 10.75% (for DHEA-d6), and while AST-d2, DHEA-d6 and 11KT-d3 were <LOQ once the internal standards were diluted 10-fold, the precision parameter could be determined for the other deuterated steroids, ranging from 5.82% (for 17OHP4-d8) to 12.48% (for 3αDIOL-d3) (supplemental Table 4). The accuracy %RSD values for the QC compounds were <6%, while the precision %RSD average values were 6.31% on day 1, 4.82% on day 2 and 5.29% on day 3. Acceptable recovery average values were also measured at 98.17% on day 1, 106.03% on day 2 and 97.31% on day 3 (supplemental Table 5).

3.2.4 Recovery, matrix effect and process efficiency of the steroid metabolites

The recovery, matrix effect and process efficiency were determined at at least three concentrations for each analyte, with most steroids being tested at four concentrations and Pdione assessed at only two concentrations. Extraction efficiency of the steroid metabolites from the serum matrix are represented by the recovery values, which ranged from 91.04% to 115.95% at 2 ng/mL, from 87.58% to 113.24% at 20 ng/mL, from 86.47% to 112.20% at 100 ng/mL and from 85.28% to 113.02% at the highest concentration tested, 200 ng/mL (Table 6). Overall, the percentage recovery of the steroid metabolites included in the method ranged from 85.28 to 115.95% (average 102.42%; %RSD, 6.50%, n=191). The matrix effect showed both ion suppression together with ion enhancement at all four concentrations tested, and ranged from -11.17% to 12.80% at 2 ng/mL, from -13.51% to 14.24% at

20 ng/mL, from -15.24% to 12.02% at 100 ng/mL and from -12.42% to 14.25% at 200 ng/mL (Table 6). Overall the matrix effect showed ion suppression at 2 ng/mL, -0.65%; ion enhancement at 20 ng/mL, 0.58%; ion suppression at 100 ng/mL, -1.76%; and ion enhancement at 200 ng/mL, 3.35%. Ion enhancement was detected only for A4, 11K5αDIONE, AST, 11βOHDHP4, A5, Pdiol, 11KT, 11OHPdione, 3α,11β-diOHDHP4 and 11OHPdiol at all the concentrations tested, while ion suppression was detected for 11KAST, 11-DHC, 21-dE, 11αOHP4, 11OHAST, corticosterone and 11OHT throughout the concentration range tested. The process efficiency was also validated and showed an overall average of 101.79% (%RSD, 7.36, n=191), ranging from 80.58% (for 11KDHP4 at 100 ng/mL) to 115.69% (for 11OH5αDIONE at 200 ng/mL). This parameter ranged from 88.48% to 116.23% at the lowest concentration tested, while at the highest concentration the process efficiency ranged from 92.62% to 115.69% (Table 6).

3.2.5 Ion ratios and accuracy bias

Ion ratios, defined as QL/QN, were validated by calculating the %RSD of the ion ratios of the standard curve for each analyte. These ratios can be routinely assessed to determine if the calibration is within range and whether a sample should be rerun. The ion ratios ranged from 0.15 to 1.73 (average 0.74) and the %RSD from 2.13 to 15.11 (average 10.5%), within acceptable limits (supplemental Table 1).

In the current study, accuracy bias was defined as the difference between a standard curve generated from an in-house steroid mix and a standard curve generated by using an independent "outside" steroid mix. Accuracy bias was determined to be acceptable, with %RSD values ranging from 3.8 to 8.12 and the comparison of the recovery of the analytes ranging from 72.05% to 102.82% (average 89.32%). 11-DOCSOL and 21-dF showed the greatest recovery at 101.59% and 102.82% respectively, while A4 and DOC showed 75.78% and 72.05% recovery, respectively (supplemental Table 6).

4. Discussion

Our UPC²-MS/MS method has established a means for a targeted steroid metabolome analysis in a serum matrix and allows the evaluation of the full complement of C_{19}, C11-oxy C_{19}, C_{21} and C11-oxy C_{21} steroids. The steroid metabolites included in this method were selected to enable analysis of comprehensive *in vivo* steroid panels that identify multiple steroid pathways active in normal physiology and disease states. MS based analytical methods that parallel our study, in terms of the inclusion of the C11-oxy steroids into analyses of large numbers of steroid metabolites are limited. A GC-MS and a GC×GC-time-of-flight MS method was recently reported in which 40 urinary steroid metabolites were quantitated with the inclusion of only three C11-oxy steroids: 11OHAST, 11Ketiocholanolone and 11OHetiocholanolone [45 and 51, respectively]. It is apt to mention that the latter two metabolites were incorrectly grouped as glucocorticoid metabolites in the aforementioned reports, with prior studies and those thereafter following suite. We have established that 11Ketiocholanolone and 11OHetiocholanolone are not downstream metabolites in the glucocorticoid pathway. Another study which incorporated a number of C11-oxy androgens -11OHA4, 11αOHA4, 11KA4, 11KT, 11KDHT and 11OHT, was carried out using LC-MS/MS quantifying 23 steroids in human plasma and prostate tissue [52]. One of the most comprehensive analysis of endogenous steroids to date, was carried out using GC-MS/MS in the identification and quantification of 100 endogenous steroids in human serum. The study included 21-dF, 11OHA4, 11OHAST (and the sulfated derivative), 11β-hydroxyepiandrosterone (and the sulfated derivative) and 11OHetiocholanolone (and the sulfated derivative) [53]. The driving force behind the development of improved analytical methods in steroid analysis is most certainly endocrinological disorders and diseases which perturb normal steroid hormone levels, exacerbating clinical conditions. The generation of comprehensive steroid profiles which also include all the C11-oxy steroids as well as the inactive C11-oxy C_{19} and C11-oxy C_{21} steroids are crucial to these analyses and have, however, been hampered in part due to many of these steroids being commercially unavailable. The current analytical method was specifically designed to include the C11-oxy steroid intermediates which would allow the clinician to track steroidogenic pathways *in vivo*.

In LC-MS/MS analysis, MRMs of analytes should not be of concern if chromatographic separation is resolved optimally. Our approach in the identification and quantitation of the steroids was, therefore, to firstly separate the steroids chromatographically and secondly by unique MRMs, if the resolution was unsatisfactory using the chosen gradient and stationary phase. While the first approach included variables which could be altered, such as the stationary phase, the mobile phase composition and the gradient (type, time and steps), in addition to the length of the total run, the second approach was to identify MRMs, including both larger and smaller molecular ions to separate specific steroids that could not be separated chromatographically. Even though we were able to achieve better separation of our steroid mix using lower flow rates —optimization from 2 mL/min to 1.3 mL/min. It should be noted that to best avoid cross-talk interference, ionisation of some steroids were compromised. While this seems counterproductive especially in cases where steroids would be present at low levels *in vivo*, compensating for cross-talk was considered more important than the peak area/ionisation in ensuring that the steroid being quantitated is the steroid in question. Indeed, when we consider that the C11-oxy steroids included in the method are similar in molecular weight and structure as well as in molecular ion species, precision in the identification of each individual

steroid is extremely important. In our hands the utilisation of SFC in steroid analysis has ultimately provided new insights into the potential of UPC²-MS/MS, with the current method highlighting its application in the identification of cross-talk between steroid metabolites. Furthermore, while more than 52 steroid metabolites could be separated under 15 min, we were also able to identify compromised reference steroid powders contaminated with low levels of their precursor steroids.

The deuterium-labelled steroids used as internal standards in this method were validated and their concentrations optimised to lie within the middle to lower concentration of the calibration range of their corresponding non-labelled steroid. In addition, cross-talk between the labelled and unlabelled standards were considered and unique MRMs established to ensure no cross-talk interference, even though two of the internal standards were labelled at only two positions. T-d2 has nevertheless also been used successfully as an internal standard in LC-MS methods [54,55]. While labelled steroids are used at different concentrations and high deuterated DHEA concentrations have previously been reported in LC-MS/MS analysis [55], the current method required DHEA-d6 as well as AST-d2, 11Ketiocholanolone-d5 and 3αDIOL-d3 to be used at higher concentrations, 1.5 to 2.5-times higher than that of T-d2 and A4-d7. Interestingly, these listed standards all possess a C3-hydroxyl group in contrast to the internal standards with C3-keto groups which required lower levels (ng on column) for inclusion in the analysis. In addition, molecular ions $[M+H]^+$ were detected for the C3-keto △4 steroids (A4, P4, 17OHP4 and T) as was also reported previously by Parr *et al*. [56], while fragments after water loss(es) were obtained for the C3-hydroxy △5 steroids (DHEA, P5, 17OHP5 and A5).

Our analysis suggest that the functional groups present on C3 and C11 appear to be better determinants in predicting steroid elution profiles and that the functional group present on C17 as well as those of C20 and C21, are not the primary determinants of the elution time of a steroid from a BEH 2-EP column. Furthermore, we observed that both the C_{19} and C_{21} steroids interact similarly with the column hybrid silica particles with specific C_{19} and C_{21} steroids eluting at the same time — 5αDIONE and DHP4, A4 and P4, 11K5αDIONE and 11KDHP4, and 11KA4 and 11KP4. Steroid metabolites included in our method with the weakest ionisation peaks and highest LOQ were: Pdione < AST, P5, 3αDIOL and 11OHAST < DHEA, 11OH5αDIONE, epiallopregnanolone, 11βOHDHP4, 11αOHDHP4, 11KPdione, A5, 11OHPdione, 3α,11β-diOHDHP4, 11KPdiol and 11OH3αDIOL. These steroids would be more readily detected in disease conditions where patient steroid output is increased due to the condition, while their levels could also be determined *in vitro*, if stimulation of steroid production is applied to model systems. It should be noted that an increase injection volume from 2 µL to 5 µL, does not lead to a linear increase in steroid quantification (data not shown; also not not advised in [11]), and while this option could be considered to increase the detection of specific steroid peaks, it would depend on the samples to be analysed. In addition, an increase of 500 µL to 1 mL sample volume would also be a viable option to increase steroid peak ionisation, this sample volume is however not always available.

In total, 32 C11-hydroxyl and C11-keto steroids were included in this method, with good accuracy and precision, and recovery, also confirmed by the process efficiency values. The following steroids had the highest ionisation peak and the lowest LOQ: P4, 16OHP4, E, 11OHT and F, with both 11OHT and F possessing a C11-hydroxyl group and E a keto-group on C11. In addition, this is the first

analytical method to simultaneously quantify intermediate metabolites of the C11-oxy backdoor pathway: 11KDHP4, 11αOHDHP4, alfaxalone, 11βOHDHP4, 3α,11β-diOHDHP4, 11OHPdione, 11OHPdiol, 11KPdione and 11KPdiol. The latter six C11-oxy steroids together with 11OH3αDIOL, 11K3αDIOL and 11OHDHT were specifically synthesised for the study. Ultimately, the 11OHA4-pathway and the C11-oxy backdoor pathway (supplemental Figure 1) can be fully analysed as all the metabolites originating from 11OHA4 and 11αOHP4, 11βOHP4, 11KP4, 21-dF and 21-dE, respectively, can be accurately quantified as intermediates and end-products. Moreover, enzyme activity of steroidogenic HSD enzymes —11βHSDs, 17βHSDs, SRD5As, 3αHSDs and RL-HSDs, and of steroidogenic P450 enzymes —CYP17A1 and CYP11B1, can be determined *in vivo* and possible deficiencies highlighted in patients. Our QC samples also included a C11-keto steroid, prednisone, which was, as previously mentioned, included to monitor extraction efficiency and the method, and four non-steroidal compounds (included to monitor the method only). The inclusion of the UPC^2 QC reference compounds, reported here for the first time using UPC^2-MS/MS, together with prednisone, ensures that the gradient and MS settings are optimal, while at the same time placing intermittent quality control checkpoints which could be determined within minutes by analysing compounds at the end of a QC sample run. As the method described in the current study is a multiplexed steroid assay, the preparation of steroid and internal standards, and QC samples could contribute to the complexity of running the method routinely. However, the steroids and internal standards, together with the QC samples, can be prepared in batches and stored at low temperatures and only removed from storage when required. Furthermore, the extraction of calibrators and samples can be carried out simultaneously 24 hrs prior to analysis, depending on sample numbers.

A pressing question with regards to the application of UPC^2-MS/MS quantification in clinical laboratories that routinely analyse human serum samples, is whether such protocols could be standardised. While the current study and others [11,13,47,48] highlight the advantages of UPC^2-MS/MS, as it currently stands, chromatographic resolution requires further optimisation, potentially not only involving the user but also the manufacturer. Communication errors, pressure irregularities determined by column usage, blockage of columns due to sample quality and low ULOQ which complicates quantification when samples analysed contain compounds present at either very low and very high levels, are some of the problems experienced when using UPC^2-MS/MS. However, low LOQs, high throughput, superior peak resolution and UPC^2 being an environmentally green method as CO_2 is used as its mobile phase, outweigh the pitfalls. Incorporation of UPC^2 systems in more laboratories could therefore pave a way for this analytical technique to become commonplace in the clinical routine setting.

While our method incorporates more than 50 steroids, many more known, and as yet, unknown, exist including but not limited to epi-steroids, C7-, C16- and C20-hydroxylated steroids. These steroids may be present in serum, not detected/quantified in our method and may potentially influence quantification due to cross-talk interference, if they are present at high levels. There are of course a number of limitations to any analytical LC-MS method which could be considered. In order to optimise the ionisation of steroid metabolites, buffer composition could be considered, as it has been shown that ionisation of steroids, which include T, P4, AST, 5αDIONE and DHP4, is improved when using a mixture of MeOH/H_2O (97.5/2.5 v/v) with 1 mM ammonium fluoride added [56]. Improved resolution has also been shown with CO_2/MeOH (90/10 v/v) containing 5 mM ammonium acetate

and 0.5% water as the mobile phase [57]. It is possible that a solid-phase extraction clean-up of samples could minimise matrix effects leading to ion suppression and enhancement [58], while the final resuspension solvent could also be optimised for better peak detection. Additional internal standards such as stable isotope labelled steroids, could also be added, once commercially available, ensuring a labelled steroid for each steroid to be analysed and quantitated.

This study has the potential to provide a platform for the incorporation of the C11-oxy steroids into analytical methods applicable to endocrinological investigations at clinical and basic science level. Assessment of the contribution of the C11-oxy steroids to the androgen pool, would thus direct investigations into the significance of these hormones in normal and abnormal physiology. Furthermore, normal reference ranges of the C11-oxy steroids can be determined using this analytical method, while deviations from these ranges could be used to categorise disease states and their severity. Its application stretches over many research fields and undefined steroid-related clinical conditions would benefit from the wide range of steroid analysis provided by this method, including PCOS and infertility, while adrenarche, menarche and puberty, onset of parturition and menopause still remain largely undefined in terms of the C11-oxy steroids. In addition, besides CRPC, other endocrine-linked cancers, such as breast and skin cancer, should also be defined in terms of the C11-hydroxyl and C11-keto derivatives of the C_{19} and C_{21} steroids. A targeted steroid analysis is therefore crucial to future diagnostic and prognostic scoring in patients, with this study laying a sound foundation.

Conflict of interest

The authors declare that there exist no conflicts.

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Appendix. Supplementary data

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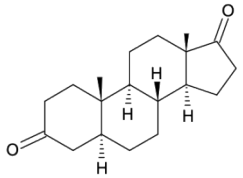
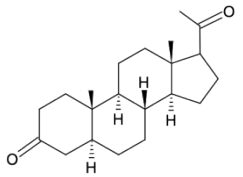
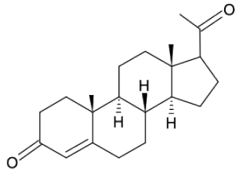
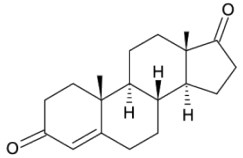
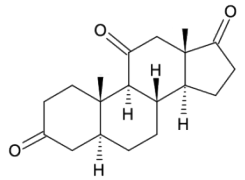
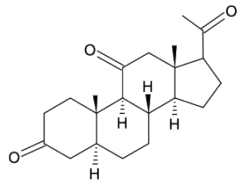
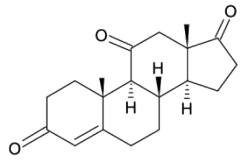
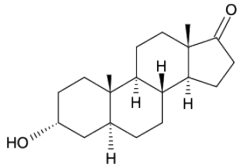
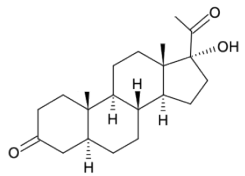
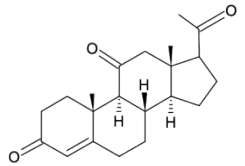
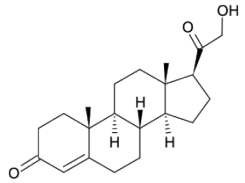
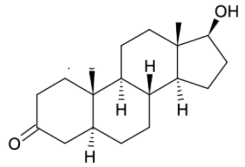
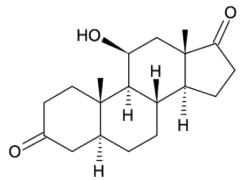
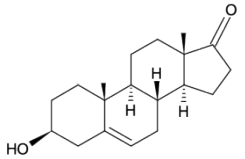
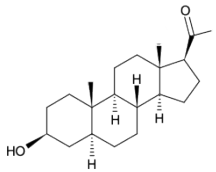
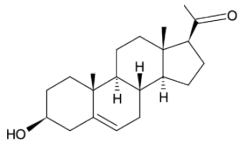
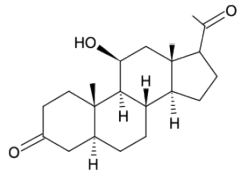
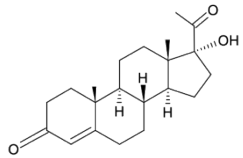
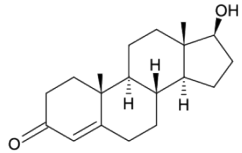
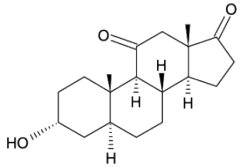
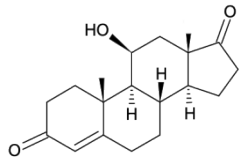
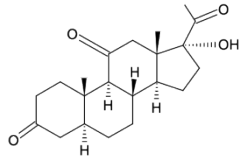
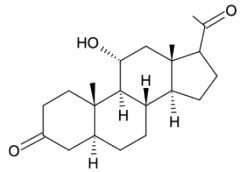
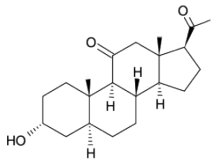
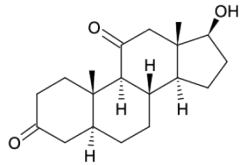
Table 1 Steroid reference standards, deuterium-labelled internal standards and quality control (QC) compounds.

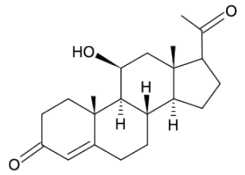
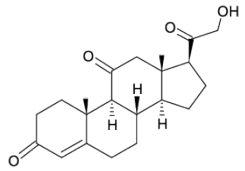
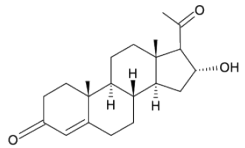
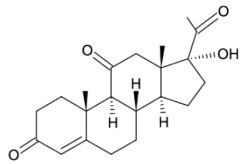
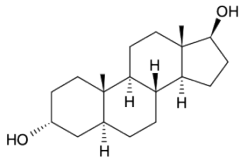
Steroid metabolites						
IUPAC name	**Trivial name/Abbreviation**	**CAS Number**	**% purity**	**Molecular weight**	**Chemical formula**	**Biochemical structure**
5α-androstan-3,17-dione	5α-androstanedione/5αDIONE	846-46-8	97	288.4	$C_{19}H_{28}O_2$	
5α-pregnan-3,20-dione	Dihydroprogesterone/DHP4	566-65-4	98	316.5	$C_{21}H_{32}O_2$	
4-pregnen-3,20-dione	Progesterone/P4	57-83-0	98	314.5	$C_{21}H_{30}O_2$	
4-androsten-3,17-dione	Androstenedione/A4	63-05-8	98	286.4	$C_{19}H_{26}O_2$	

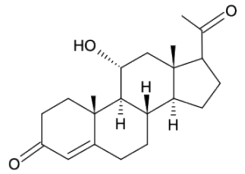
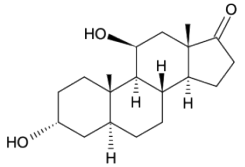
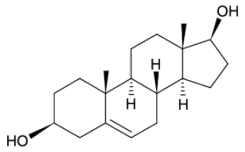
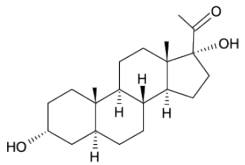
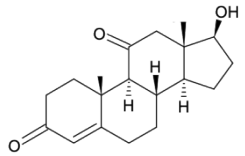
5α-androstan-3,11,17-trione	11K5αDIONE	1482-70-8	98	302.4	C19 H26O3	
5α-pregnan-3,11,20-trione	11keto-dihydroprogesterone/11KDHP4	2089-06-7	98	330.5	C21H30O3	
4-androsten-3,11,17-trione	11keto-androstenedione/11KA4	382-45-6	98	300.4	C19H24O3	
5α-androstan-3α-ol-17-one	Androsterone/AST	53-41-8	98	290.4	C19H30O2	
5α-pregnan-17α-ol-3,20-dione	Pdione	570-59-2	98	332.5	C21H32O3	

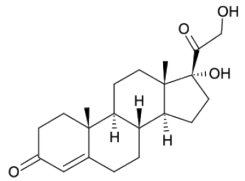
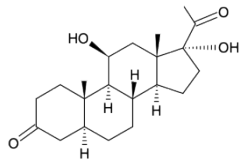
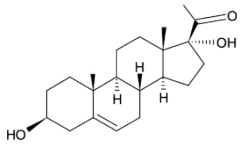
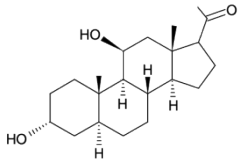
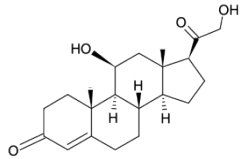
4-pregnen-3,11,20-trione	11keto-progesterone/11KP4	516-15-4	98	328.5	C21H28O3	
4-pregnen-21-ol,3,20-dione	Deoxycorticosterone/DOC	64-85-7	99	330.5	C21H30O3	
5α-androstan-17β-ol-3-one	Dihydrotestosterone/DHT	521-18-6	97.5	290.4	C19H30O2	
5α-androstan-11β-ol-3,17-dione	11OH5αDIONE	N/A	98	304.4	C19H28O3	
5-androsten-3β-ol-17-one	Dehydroepiandrosterone/DHEA	53-43-0	99	288.4	C19H28O2	

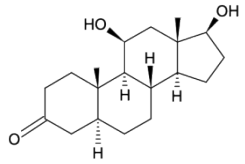
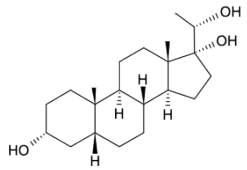
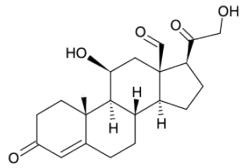
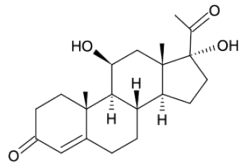
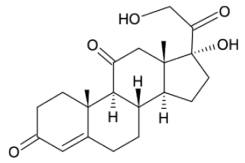
5α-pregnan-3β-ol-20-one	Epiallopregnanolone	516-55-2	-	318.5	$C_{21}H_{34}O_2$	
5-pregnen-3β-ol-20-one	Pregnenolone/P5	145-13-1	98	316.5	$C_{21}H_{32}O_2$	
5α-pregnan-11β-ol-3,20-dione	11β-hydroxydihydroprogesterone/11βOHDHP4	N/A	98	332.5	$C_{21}H_{32}O_3$	
4-pregnen-17α-ol-3,20-dione	17α-hydroxyprogesterone/17OHP4	604-09-1	95	330.5	$C_{21}H_{30}O_3$	
4-androsten-17β-ol-3-one	Testosterone/T	58-22-0	99	288.4	$C_{19}H_{28}O_2$	
5α-androstan-3α-ol-11,17-dione	11keto-androsterone/11KAST	1231-82-9	98	304.4	$C_{19}H_{28}O_3$	

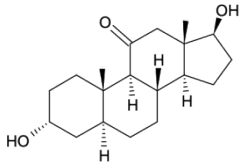
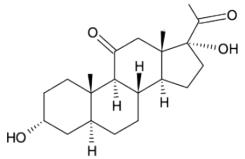
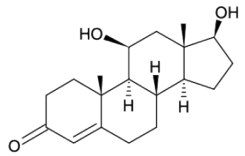
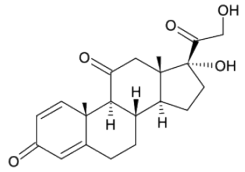
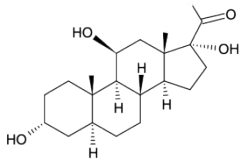
4-androsten-11β-ol-3,17-dione	11β-hydroxyandrostenedione/11OHA4	382-44-5	98	302.4	$C_{19}H_{26}O_3$	
5α-pregnan-17α-diol-3,11,20-trione	11KPdione	N/A	98	346.5	$C_{21}H_{30}O_4$	
5α-pregnan-11α-ol-3,20-dione	11α-hydroxydihydroprogesterone/11αOHDHP4	N/A	98	332.5	$C_{21}H_{32}O_3$	
5α-pregnan-3α-ol-11,20-dione	Alfaxalone	23930-19-0	98	332.5	$C_{21}H_{32}O_3$	
5α-androstan-17β-ol-3, 11-dione	11keto-dihydrotestosterone/11KDHT	32694-37-4	98	304.4	$C_{19}H_{28}O_3$	

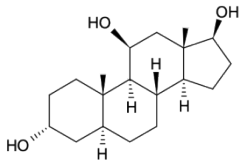
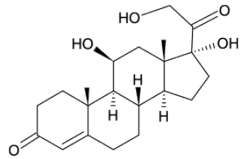
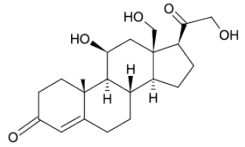
4-pregnen-11β-ol-3,20-dione	11β-hydroxyprogesterone/11βOHP4	600-57-7	98	330.5	$C_{21}H_{30}O_3$	
4-pregnen-21-ol-3,11,20-trione	11-dehydrocorticosterone/11-DHC	72-23-1	98	344.5	$C_{21}H_{28}O_4$	
4-pregnen-16α-ol-3,20-dione	16α-hydroxyprogesterone/16OHP4	438-07-3	97	330.5	$C_{21}H_{30}O_3$	
4-pregnen-17-ol-3,11,20-trione	21-deoxycortisone/21-dE	1882-82-2	98	344.4	$C_{21}H_{28}O_4$	
5α-androstan-3α,17β-diol	3αDIOL	1852-53-5	98	292.5	$C_{19}H_{32}O_2$	

4-pregnen-11α-ol-3,20-dione	11α-hydroxyprogesterone/11αOHP4	80-75-1	98	330.5	C21H30O3	
5α-androstan-3α,11β-diol-17-one	11β-hydroxyandrosterone/11OHAST	57-61-4	98	306.4	C19H30O3	
5-androsten-3β,17β-diol	Androstenediol/A5	521-17-5	98	290.4	C19H30O2	
5α-pregnan-3α,17α-diol-20-one	Pdiol	6890-65-9	98	334.5	C21H34O3	
4-androsten-17β-ol-3,11-dione	11keto-testosterone/11KT	564-35-2	98	302.4	C19H26O3	

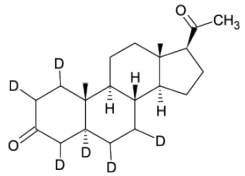
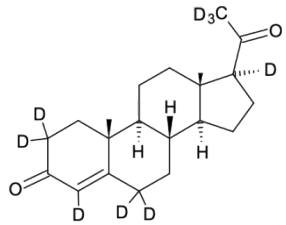
4-pregnen-17α,21-diol-3,20-dione	11-deoxycortisol/11-DOCSOL	152-58-9	98	346.5	$C_{21}H_{30}O_4$	
5α-pregnan-11β,17α-diol-3,20-dione	11OHPdione	N/A	98	348.5	$C_{21}H_{32}O_4$	
5-pregnen-3β,17α-diol-20-one	17α-hydroxypregnenolone/17OHP5	387-79-1	96	332.5	$C_{21}H_{32}O_3$	
5α-pregnan-3α, 11β-diol-20-one	3α,11β-diOHDHP4	N/A	98	348.5	$C_{21}H_{34}O_3$	
4-pregnen-11β,21-diol-3,20-dione	Corticosterone	50-22-6	95	346.5	$C_{21}H_{30}O_4$	

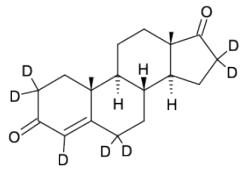
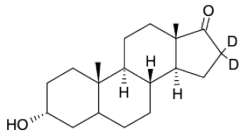
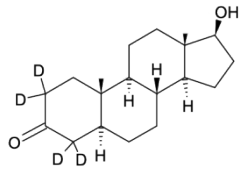
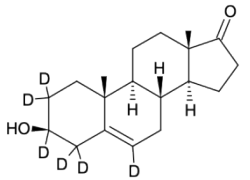
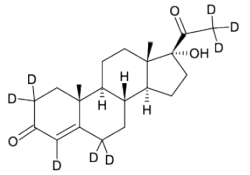
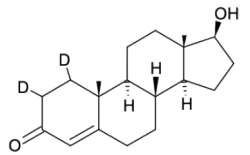
5α-androstan-11β,17β-diol-3, 11-dione	11β-hydroxydihydrotestosterone/11OH DHT	N/A	98	306.4	$C_{19}H_{30}O_3$	
5β-pregnan-3α,17,20α-triol	Pregnanetriol	1098-45-9	98	336.5	$C_{21}H_{36}O_3$	
4-pregnen-11β,21-diol-3,18,20-trione	Aldosterone	52-39-1	95	360.4	$C_{21}H_{28}O_5$	
4-pregnen-11β,17-diol-3, 20-dione	21-deoxycortisol/21-dF	641-77-0	98	346.5	$C_{21}H_{30}O_4$	
4-pregnen-17,21-diol-3,11,20-trione	Cortisone/E	53-06-5	98	360.4	$C_{21}H_{28}O_5$	

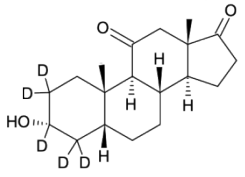
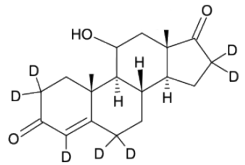
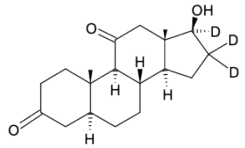
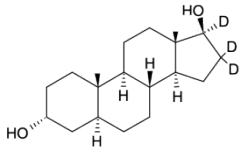
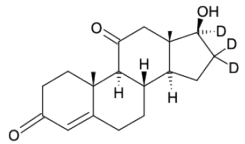
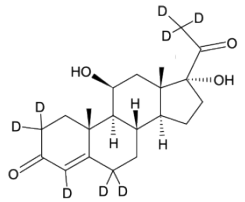
5α-androstan-11-one-3α, 17β-diol	11K3αDIOL	N/A	98	306.4	$C_{19}H_{30}O_3$	
5α-pregnan-3α, 17α-diol-11,20-dione	11KPdiol	N/A	98	348.5	$C_{21}H_{32}O_4$	
4-androsten-11β,17β-diol-3-one	11β-hydroxytestosterone/11OHT	1816-85-9	98	304.4	$C_{19}H_{28}O_3$	
1,4-pregnadien-17α,21-diol-3,11,20-trione	Prednisone	53-03-2	98	358.4	$C_{21}H_{26}O_5$	
5α-pregnan-3α,11β,17α-triol-20-one	11OHPdiol	N/A	90	350.5	$C_{21}H_{34}O_4$	

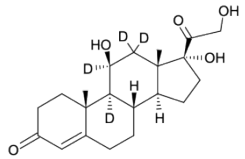
5α-androstan-3α,11β,17β-triol	11OH3αDIOL	N/A	98	308.5	$C_{19}H_{32}O_3$	
4-pregnen-11β,17,21-triol-3,20-dione	Cortisol/F	50-23-7	98	362.5	$C_{21}H_{30}O_5$	
4-pregnen-11β,18,21-triol-3,20-dione	18-hydroxycorticosterone/18OHcorticosterone	561-65-9	97	362.5	$C_{21}H_{30}O_5$	

Deuterium-labelled internal reference standards

IUPAC Name	Abbreviation	Labelled CAS Number	% purity	Molecular weight	Chemical formula	Biochemical structure
5α-pregnan-3,20-dione (1,2,4,5,6,7-D6)	DHP4-d6	N/A	95	322.5	$C_{21}D_6H_{26}O_2$	
4-pregnen-3,20-dione (2,2,4,6,6,17A,21,21,21-D9)	P4-d9	15775-74-3	98	323.5	$C_{21}D_9H_{21}O_2$	

4-androsten-3,17-dione (2,2,4,6,6,16,16-D7)	A4-d7	67034-85-9	97	293.4	$C_{19}D_7H_{19}O_2$	
5α-androstan-3α-ol-17-one (16,16-D2)	AST-d2	53-41-8	98.9	292.4	$C_{19}D_2H_{28}O_2$	
5α-androstan-17β-ol-3-one (2,2,4,4-D4)	DHT-d4	5295-66-9	98.6	294.4	$C_{19}D_4H_{26}O_2$	
5-androsten-3β-ol-17-one (2,2,3,4,4,6-D6)	DHEA-d6	1261254-39-0	98.8	294.4	$C_{19}D_6H_{22}O_2$	
4-pregnen-17α-ol-3,20-dione (2,2,4,6,6,21,21,21-D8)	17OHP4-d8	850023-80-2	98	338.5	$C_{21}D_8H_{22}O_3$	
4-androsten-17β-ol-3-one (1,2-D2)	T-d2	204244-83-7	98.2	290.4	$C_{19}D_2H_{26}O_2$	

5α-androstan-3β-ol-11,17-dione (9,12,12,16,16-D5)	11Ketiocholanolone-d5	N/A	91.6	309.4	$C_{19}D_5H_{23}O_3$	
4-androsten-11β-ol-3,17-dione (2,2,4,6,6,16,16-D7)	11OHA4-d7	N/A	98	309.4	$C_{19}D_7H_{19}O_3$	
5α-androstan-17β-ol-3, 11-dione (16,16,17A-D3)	11KDHT-d3	N/A	98	307.4	$C_{19}D_3H_{25}O_3$	
5α-androstan-3α,17β-diol (16,16,17A-D3)	3αDIOL-d3	N/A	99.4	295.4	$C_{19}D_3H_{29}O_2$	
4-androsten-17β-ol-3,11-dione (16,16,17A-D3)	11KT-d3	N/A	98	305.4	$C_{19}D_3H_{23}O_3$	
4-pregnen-11β,17-diol-3, 20-dione (2,2,4,6,6,21,21,21-D8)	21dF-d8	N/A	97	354.5	$C_{21}D_8H_{22}O_4$	

4-pregnen-11β,17,21-triol-3,20-dione (9,11,12,12-D4)	F-d4	73565-87-4	97.5	366.5	$C_{21}D_4H_{26}O_5$	

QC compounds

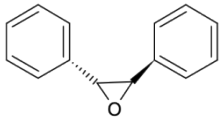
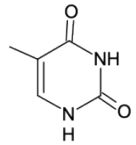
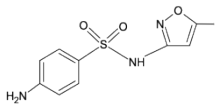
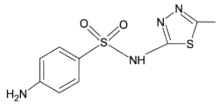
	Trivial name	CAS Number	Molecular weight	Chemical formula	Biochemical structure
	(+/-) Trans-stilbene oxide	1439-07-2	196.2	$C_{14}H_{12}O$	
	Thymine	65-71-4	126.1	$C_5H_6N_2O_2$	
	Sulfamethoxazole	723-46-6	253.3	$C_{10}H_{11}N_3O_3S$	
	Sulfamethizole	144-82-1	270.3	$C_9H_{10}N_4O_2S_2$	

Table 2 Gradient specifications of the UPC^2 separation of steroid metabolites (solvent A, CO_2; solvent B, methanol).

Step	Time (min)	Flow (mL/min)	Solvent A (%)	Solvent B (%)	Curve
1	0	1.3	96	4	Initial
2	3.3	0.65	95.1	4.9	6
3	8	0.65	94.8	5.2	6
4	12.2	0.95	89	11	3
5	12.7	1.3	75	25	6
6	13	1.3	75	25	6
7	13.2	1.3	96	4	6
8	15	1.3	96	4	6

Table 3 UPC²-MS/MS parameters for the detection and quantification of steroid standards, internal deuterated standards and QC compounds. Retention time (RT); internal reference standard used to quantify each analyte; molecular ion species and MRM mass transitions; and mass spectrometer settings: cone voltage (CV) and collision energy (CE).

Steroid metabolites*	RT (min)	Internal reference standard	Mass transitions			CV (V)			CE (eV)		
			Quantifier	Qualifier	Qualifier						
5αDIONE	1.21	DHP4-d6	289.2 > 253.1	289.2 > 271.2	289.2 > 97.2	30	30	30	15	15	22
DHP4	1.24	DHP4-d6	317.0 > 95.0	317.0 > 105.2	317.0 > 175.2	30	30	30	30	30	20
P4	1.77	P4-d9	315.2 > 79.15	315.2 > 297.0	315.2 > 123.04	28	28	28	35	20	35
A4	1.82	A4-d7	287.2 > 123.0	287.2 > 173.0	287.2 > 211.1	30	30	30	25	20	20
11K5αDIONE	1.97	A4-d7	303.2 > 241.0	303.2 > 248.9	303.2 > 227.1	35	35	35	20	15	20
11KDHP4	2.12	A4-d7	331.2 > 277.3	331.2 > 255.0	331.2 > 268.8	25	25	25	15	20	20
11KA4	3.20	AST-d2	301.2 > 121.1	301.2 > 265.21	301.2 > 257.0	35	35	35	30	15	25
AST	3.29	AST-d2	273.2 > 185.1	273.2 > 69.07	273.2 > 55.14	30	30	30	20	30	30
11KP4	3.33	AST-d2	329.21 > 121.0	329.21 > 285.11	329.21 > 84.8	15	15	15	20	20	20
Pdione	3.52	DHT-d4	333.4 > 137.26	333.4 > 70.82	333.4 > 228.76	20	20	20	25	25	25
DOC	3.56	DHT-d4	331.18 > 159.17	331.18 > 130.89	331.18 > 117.02	28	28	28	22	40	30
DHT	3.68	DHT-d4	291.0 > 255.29	291.0 > 104.9	291.0 > 159.0	30	30	30	15	30	25
Epiallopregnanolone	4.21	DHEA-d6	301.4 > 189.2	301.4 > 283.15	301.4 > 80.9	30	30	30	20	15	30
DHEA	4.24	DHEA-d6	271.2 > 253.2	271.2 > 213.0	271.2 > 91.05	30	30	30	15	15	40
11OH5αDIONE	4.30	DHEA-d6	305.0 > 269.2	305.0 > 211.0	305.0 > 97.2	35	35	35	15	20	30
P5	4.49	DHEA-d6	299.4 > 80.97	299.4 > 131.0	317.24 > 158.9	16	16	16	20	25	20
11βOHDHP4	5.35	17OHP4-d8	333.1 > 85.0	333.1 > 159.0	333.1 > 279.25	30	30	30	20	20	15
17OHP4	5.78	17OHP4-d8	331.1 > 122.9	331.1 > 295.0	331.1 > 312.8	26	26	26	25	15	15
T	6.15	T-d2	289.2 > 123.0	289.2 > 271.18	289.2 > 186.8	30	30	30	25	20	20
11KAST	6.17	11Ketiocholanolone-d5	287.0 > 229.1	287.0 > 147.2	287.0 > 211.25	30	30	30	20	30	20
11OHA4	7.12	11OHA4-d7	303.2 > 267.2	303.2 > 285.2	303.2 > 121.1	30	30	30	15	15	25
11KPdione	7.14	11OHA4-d7	347.4 > 329.0	347.4 > 311.1	347.4 > 251.0	25	25	25	15	15	20
11αOHDHP4	7.38	11OHA4-d7	333.0 > 297.3	333.0 > 85.2	333.0 > 279.1	30	30	30	15	20	15
Alfaxalone	7.82	11KDHT-d3	333.2 > 214.96	333.2 > 85.0	333.2 > 297.0	25	25	25	15	25	15
11KDHT	8.20	11KDHT-d3	305.2 > 251.0	305.2 > 144.9	305.2 > 118.8	30	30	30	15	30	30
11βOHP4	8.97	3αDIOL-d3	331.2 > 295.01	331.2 > 313.0	331.2 > 121.15	30	30	30	15	15	25
11-DHC	9.16	3αDIOL-d3	345.3 > 121.15	345.3 > 163.09	345.3 > 301.2	30	30	30	25	25	30
16OHP4	9.50	3αDIOL-d3	331.2 > 97.01	331.2 > 109.1	331.2 > 313.1	30	30	30	20	30	15
3αDIOL	9.66	3αDIOL-d3	275.2 > 257.25	275.2 > 175.0	275.2 > 81.1	15	15	15	15	15	30
21-dE	9.95	3αDIOL-d3	345.2 > 163.2	345.2 > 121.08	345.2 > 105.0	25	25	25	25	30	35
11αOHP4	10.07	3αDIOL-d3	331.2 > 295.2	331.2 > 313.04	331.2 > 120.9	30	30	30	15	15	25

11OHAST	10.29	11KT-d3	289.0 > 271.3	289.0 > 253.2	289.0 > 213.0	15	15	15	15	15	15
Pdiol	10.33	11KT-d3	217.4 > 111.0	217.4 > 299.0	217.4 > 93.15	20	20	20	25	15	30
A5	10.35	11KT-d3	273.09 > 201.17	273.09 > 185.28	273.09 > 129.14	20	20	20	15	15	30
11KT	10.47	11KT-d3	303.2 > 147.1	303.2 > 285.0	303.2 > 107.2	30	30	30	25	15	30
11-DOCSOL	10.74	11KT-d3	247.3 > 123.04	247.3 > 78.94	247.3 > 80.97	30	30	30	30	35	35
11OHPdione	10.78	11KT-d3	349.2 > 114.99	349.2 > 183.25	349.2 > 71.03	25	25	25	20	30	30
17OHP5	10.80	11KT-d3	397.2 > 159.12	397.2 > 133.1	397.2 > 147.3	32	32	32	26	24	25
3α,11β-diOHDHP4	10.98	11KT-d3	299.4 > 159.0	299.4 > 95.0	299.4 > 119.1	30	30	30	25	30	30
Corticosterone	11.12	11KT-d3	347.2 > 121.1	347.2 > 104.98	347.2 > 97.1	26	26	26	25	35	25
11OHDHT	11.20	21-dF-d8	307.0 > 271.0	307.0 > 213.1	307.0 > 253.0	25	25	25	15	20	20
Pregnanetriol	11.61	21-dF-d8	301.2 > 283.2	301.2 > 187.0	301.2 > 135.01	25	25	25	15	15	20
Aldosterone	11.65	21-dF-d8	361. 0 > 343.0	361. 0 > 315.0	361. 0 > 97.0	30	30	30	18	20	32
21-dF	11.92	21-dF-d8	347.1 > 147.27	347.1 > 210.9	347.1 > 95.39	25	25	25	20	20	40
E	11.95	21-dF-d8	361.2 > 163.16	361.2 > 120.94	361.2 > 105.19	30	30	30	20	30	30
11K-3αDIOL	11.97	21-dF-d8	307.2 > 271.0	307.2 > 253.0	307.2 > 159.13	20	20	20	15	20	20
11KPdiol	11.99	21-dF-d8	331.2 > 289.24	331.2 > 271.32	331.2 > 253.05	30	30	30	15	15	20
11OHT	12.34	21-dF-d8	305.3 > 269.0	305.3 > 121.0	305.3 > 105.1	35	35	35	15	20	20
Prednisone	12.41	21-dF-d8	359.0 > 147.0	359.0 > 171.0	-	25	25	-	35	35	-
11OHPdiol	13.13	F-d4	296.9 > 279.1	296.9 > 145.1	296.9 > 105.12	25	25	25	15	30	40
11OH3αDIOL	13.14	F-d4	273.0 > 107.0	273.0 > 147.2	273.0 > 161.0	25	25	25	20	20	20
F	13.15	F-d4	363.3 > 121.08	363.3 > 308.98	363.3 > 105.12	30	30	30	20	20	35
18OHCORT	13.28	F-d4	363.2 > 269.2	363.2 > 147.0	363.2 > 251.2	30	30	30	15	22	20
DHP4-d6	1.23	-	323.17 > 85.0	323.17 > 287.07	323.17 > 305.0	35	35	35	15	15	15
P4-d9	1.74	-	324.2 > 107.15	324.2 > 134.95	324.2 > 194.24	30	30	30	30	30	25
A4-d7	1.78	-	294.3 > 234.0	294.3 > 118.9	294.3 > 121.15	25	25	25	20	30	30
AST-d2	3.20	-	275.0 > 215.1	275.0 > 67.0	275.0 > 54.95	25	25	25	15	30	30
DHT-d4	3.57	-	295.2 > 105.05	295.2 > 163.19	295.2 > 215.3	30	30	30	30	20	20
DHEA-d6	4.16	-	277.3 > 219.2	277.3 > 97.2	277.3 > 202.1	25	25	25	15	20	20
17OHP4-d8	5.60	-	340.1 > 322.0	340.1 > 168.2	340.1 > 147.2	26	26	26	15	15	25
T-d2	6.09	-	291.0 > 201.3	291.0 > 164.84	291.0 > 105.19	30	30	30	20	20	15
11Ketiocholanolone-d5	6.41	-	291.3 > 230.2	291.3 > 273.24	291.3 > 92.97	25	25	25	20	15	30
11OHA4-d7	7.12	-	310.1 > 128.02	310.1 > 168.4	310.1 > 134.95	25	25	25	25	30	30
11KDHT-d3	8.15	-	308.2 > 290.12	308.2 > 131.05	308.2 > 197.15	25	25	25	15	30	20
3αDIOL-d3	9.62	-	260.2 > 178.1	260.2 > 135.1	260.2 > 109.1	25	25	25	15	15	20
11KT-d3	10.46	-	306.2 > 171.0	306.2 > 109.24	306.2 > 212.95	15	15	15	30	30	20
21-dF-d8	11.85	-	355.03 > 319.28	355.03 > 125.15	355.03 > 180.24	20	20	20	15	25	20
F-d4	13.14	-	367.26 > 273.21	367.26 > 123.04	367.26 > 109.04	30	30	30	20	30	35
(+/-) Trans-stilbene oxide	0.62	-	197.19 > 105.12	197.19 > 76.99	-	25	25	-	20	30	-

Thymine	6.2	-	127.05 > 53.97	127.05 > 55.95	-	25	25	-	20	20	-
Sulfamethoxazole	13.2	-	254.06 > 108.06	254.06 >92.17	-	30	30	-	25	30	-
Sulfamethizole	13.7	-	271.032 > 107.99	271.032 > 92.17	-	30	30	-	20	30	-

*IUPAC names of the analytes are described in Table 1.

Table 4 LOD, LOQ, calibration range in ng/mL and linearity (r^2) of the calibration range of the steroids and QC samples.

Steroid metabolite	**LOD**		**LOQ**		**Calibration range**	**r^2**
	ng/mL	**pg on column**	**ng/mL**	**pg on column**		
5αDIONE	0.1	0.67	1	6.67	1 - 1000	0.9998
DHP4	0.2	1.33	1	6.67	0.2 - 1000	0.9999
P4	0.02	0.133	0.1	0.67	0.02 - 1000	0.9999
A4	0.2	1.33	1	6.67	1 - 1000	0.9994
11K-5αDIONE	1	6.67	2	13.33	1 - 1000	0.9974
11KDHP4	0.2	1.33	1	6.67	1 - 1000	0.9992
11KA4	0.2	1.33	1	6.67	0.2 - 1000	0.9999
AST	10	66.67	20	133.33	10 - 1000	0.9998
Pdione	20	133.33	100	666.67	20 - 1000	0.9999
11KP4	0.2	1.33	1	6.67	0.2 - 1000	0.9999
DOC	1	6.67	1	6.67	1 - 1000	0.9999
DHT	0.1	0.67	0.2	1.33	0.2 - 1000	0.9997
11OH5αDIONE	2	13.33	10	66.67	2 - 1000	0.9997
DHEA	2	13.33	10	66.67	2 - 1000	0.9998
Epiallopregnanolone	1	6.67	2	13.33	2 - 1000	0.9996
P5	2	13.33	10	66.67	10 - 1000	0.9996
11βOHDHP4	2	13.33	10	66.67	2 - 1000	0.9999
17OHP4	0.2	1.33	1	6.67	1 - 1000	0.9999
T	0.1	0.67	0.2	1.33	0.1 - 1000	0.9992
11KAST	1	6.67	2	13.33	1 - 1000	0.9998
11OHA4	0.2	1.33	1	6.67	1 - 1000	0.9998
11KPdione	2	13.33	10	66.67	2 - 1000	0.9998
11αOHDHP4	2	13.33	10	66.67	2 - 1000	0.9993
Alfaxalone	0.2	1.33	1	6.67	1 - 1000	0.9996
11KDHT	0.2	1.33	1	6.67	1 - 1000	0.9999
11βOHP4	0.2	1.33	1	6.67	0.2 - 1000	0.9986
11-DHC	0.2	1.33	1	6.67	1 - 1000	0.9999
16OHP4	0.02	0.133	0.1	0.67	0.02 - 1000	0.9999
21-dE	0.1	0.67	0.2	1.33	0.1 - 1000	0.9997

3αDIOL	10	66.67	20	133.33	10 - 1000	0.9992
11αOHP4	0.1	0.67	1	6.67	0.1 - 1000	0.9998
11OHAST	10	66.67	10	66.67	10 - 1000	0.9998
A5	2	13.33	10	66.67	10 - 1000	0.9993
Pdiol	1	6.67	2	13.33	1 - 1000	0.9997
11KT	0.2	1.33	1	6.67	1 - 1000	0.9998
11-DOCSOL	0.2	1.33	1	6.67	0.2 - 1000	0.9998
11OHPdione	2	13.33	10	66.67	2 - 1000	0.9997
17OHP5	2	13.33	10	66.67	2 - 1000	0.9998
3α,11β-diOHDHP4	1	6.67	2	13.33	2 - 1000	0.9999
Corticosterone	0.2	1.33	1	6.67	0.2 - 1000	0.9998
11OHDHT	0.2	1.33	1	6.67	1 - 1000	0.9999
Pregnanetriol	1	6.67	2	13.33	1 - 1000	0.9995
Aldosterone	1	6.67	2	13.33	1 - 1000	0.9995
21-dF	0.2	1.33	1	6.67	1 - 1000	0.9998
E	0.02	0.133	0.1	0.67	0.02 - 1000	0.9996
11K3αDIOL	0.2	1.33	1	6.67	0.2 - 1000	0.9994
11KPdiol	0.2	1.33	1	6.67	1 - 1000	0.9995
11OHT	0.02	0.133	0.1	0.67	0.02 - 1000	0.9997
Prednisone	0.2	1.33	1	6.67	0.2 - 1000	0.9981
11OHPdiol	1	6.67	2	13.33	2 - 1000	0.9998
11OH3αDIOL	10	66.67	20	133.33	10-1000	0.9995
F	0.002	0.013	0.02	0.133	0.002 - 1000	0.9999
18OHcorticosterone	0.2	1.33	1	6.67	0.2 - 1000	0.9995
QC compounds						
(+/-) Trans-stilbene oxide	3.75	7.5	7.5	15	7.5-750	0.9992
Thymine	3.75	7.5	7.5	15	7.5-750	0.9997
Sulfamethoxazole	<0.075	<0.15	0.075	0.15	0.075-750	0.9972
Sulfamethizole	0.75	1.5	0.75	1.5	0.75-750	0.9997

Table 5 Method validation data: accuracy (n=8) and precision with inter-day variability (n=8) calculated from response values for steroid metabolites and from area values for deuterated steroids and QC compounds.

Steroid metabolite	Accuracy (%RSD)				Precision (%RSD)											
					Day 1				Day 2				Day 3			
	ng/mL (pg on the column)				ng/mL (pg on the column)											
	2 (4)	20 (40)	100 (200)	200 (400)	2 (4)	20 (40)	100 (200)	200 (400)	2 (4)	20 (40)	100 (200)	200 (400)	2 (4)	20 (40)	100 (200)	200 (400)
5αDIONE	7.93	1.03	0.70	3.06	7.05	2.07	2.96	2.96	8.36	1.64	0.76	2.16	9.32	3.68	1.08	0.43
DHP4	6.25	5.99	5.47	3.46	11.49	5.63	3.63	5.88	7.10	4.17	2.25	5.84	12.85	4.43	1.48	3.33
P4	3.09	2.58	4.23	1.92	9.26	4.94	3.55	6.77	3.13	4.24	4.30	1.76	7.36	3.77	2.69	2.15
A4	8.11	3.28	2.20	1.51	5.09	4.18	6.46	3.71	5.91	1.78	2.53	7.53	3.39	1.34	2.46	3.32
11K5αDIONE	8.78	5.51	0.91	0.52	10.10	9.97	6.87	6.79	12.30	2.94	2.26	1.01	13.82	2.77	4.64	3.66
11KDHP4	12.77	6.10	4.63	3.43	7.95	10.73	9.80	8.43	10.01	3.14	3.26	3.86	13.93	4.12	8.46	8.58
11KA4	0.64	0.47	1.18	0.51	9.85	9.58	4.38	5.14	0.28	0.58	1.31	0.68	1.70	1.85	1.25	1.66
AST	<LOQ	5.19	2.45	1.50	<LOQ	4.61	7.59	6.76	<LOQ	2.10	3.39	0.96	<LOQ	4.03	1.16	2.45
Pdione	<LOQ	<LOQ	4.75	2.11	<LOQ	<LOQ	9.64	7.16	<LOQ	<LOQ	6.43	3.11	<LOQ	<LOQ	5.72	4.43
11KP4	0.90	1.59	0.98	2.01	5.95	7.14	6.13	4.96	2.23	2.65	1.15	4.25	9.35	5.65	1.40	3.80
DOC	<LOQ	2.74	1.80	2.20	<LOQ	6.20	8.37	3.64	<LOQ	3.92	5.10	2.06	<LOQ	3.90	3.08	3.58
DHT	1.29	1.33	1.78	1.16	8.34	4.42	4.64	4.85	1.67	0.91	2.22	0.95	7.15	2.16	2.20	2.34
11OH5αDIONE	<LOQ	4.29	0.96	1.55	<LOQ	8.38	5.88	8.38	<LOQ	4.45	1.26	1.77	<LOQ	2.50	3.14	5.89
DHEA	<LOQ	4.45	3.04	1.49	<LOQ	11.12	4.09	5.15	<LOQ	3.69	1.28	2.66	<LOQ	3.20	2.07	1.61
Epiallopregnanolone	6.02	2.03	1.17	3.99	9.49	6.96	5.80	6.34	3.97	2.24	2.27	2.75	9.48	6.16	7.01	8.89
P5	<LOQ	3.88	0.85	2.09	<LOQ	7.24	2.15	6.64	<LOQ	2.37	3.88	1.22	<LOQ	3.85	4.21	7.00
11βOHDHP4	<LOQ	4.25	1.55	1.63	<LOQ	12.18	4.76	4.31	<LOQ	5.06	1.03	1.28	<LOQ	6.09	2.49	1.97
17OHP4	1.15	0.90	0.95	2.81	5.02	3.60	5.63	1.72	3.19	0.40	1.93	1.91	4.10	2.58	2.19	4.33
T	1.33	0.94	0.91	0.50	10.03	5.04	2.57	1.79	2.33	0.29	2.25	0.75	8.12	2.80	3.93	1.39
11KAST	<LOQ	5.31	1.26	0.45	<LOQ	9.17	6.75	7.57	<LOQ	4.01	1.41	0.82	<LOQ	5.04	1.77	1.87
11OHA4	1.32	0.76	0.87	0.83	14.08	5.07	4.83	3.37	0.83	0.75	1.88	0.66	5.85	3.53	2.12	2.94
11KPdione	<LOQ	1.39	3.07	1.37	<LOQ	6.01	4.17	6.24	<LOQ	3.80	2.70	1.76	<LOQ	6.54	4.71	4.60
11αOHDHP4	<LOQ	2.17	0.66	2.59	<LOQ	7.83	2.11	7.13	<LOQ	2.09	4.17	3.39	<LOQ	8.07	10.68	11.16
Alfaxalone	9.25	3.83	1.79	5.66	8.83	9.37	5.21	7.12	10.98	2.33	2.31	3.67	11.37	9.21	10.99	7.82
11KDHT	4.36	0.69	1.31	1.62	11.17	4.44	3.88	3.13	2.67	2.34	1.96	1.08	4.68	1.46	2.55	3.24
11βOHP4	2.94	1.10	1.67	1.53	5.45	4.64	4.33	5.05	1.76	1.79	0.69	3.57	4.66	6.12	8.49	5.96
11-DHC	1.41	2.46	2.23	4.90	6.22	3.63	2.77	11.69	4.16	1.91	1.34	3.67	10.62	8.65	5.03	9.93
16OHP4	1.06	1.81	1.46	0.72	3.53	6.24	3.74	6.51	0.52	0.78	1.17	0.42	5.74	8.33	7.92	5.31
21-dE	1.54	1.48	2.61	1.15	7.11	7.56	3.11	8.83	3.17	1.67	1.55	0.72	6.74	8.27	7.92	6.16
3αDIOL	<LOQ	4.81	1.95	1.09	<LOQ	7.62	3.18	3.90	<LOQ	4.63	1.94	0.86	<LOQ	4.06	2.37	4.12
11αOHP4	3.04	0.97	0.74	1.79	3.50	5.95	3.14	7.11	2.33	0.92	1.59	0.69	6.79	9.10	6.15	6.56

11OHAST	<LOQ	7.02	4.49	1.53	<LOQ	9.09	6.58	3.44	<LOQ	10.79	5.31	1.26	<LOQ	7.06	5.73	3.54
A5	<LOQ	3.95	4.88	2.83	<LOQ	7.56	8.46	8.83	<LOQ	11.29	2.12	2.16	<LOQ	7.01	5.00	3.19
Pdiol	<LOQ	5.18	2.48	1.14	<LOQ	1.85	8.13	10.97	<LOQ	3.81	2.24	1.55	<LOQ	5.07	3.87	7.49
11KT	1.99	1.92	1.35	1.93	12.55	4.02	5.22	4.51	3.13	1.16	1.18	1.07	4.72	2.88	4.18	1.49
11-DOCSOL	2.27	2.55	1.10	3.93	14.55	8.00	8.37	5.71	2.46	3.00	4.63	1.96	10.16	5.22	8.05	3.96
11OHPdione	<LOQ	9.30	1.97	3.64	<LOQ	15.32	10.29	10.41	<LOQ	7.64	2.32	5.87	<LOQ	12.54	6.91	4.61
17OHP5	<LOQ	2.90	1.60	1.14	<LOQ	10.14	6.25	5.72	<LOQ	5.30	2.48	0.58	<LOQ	4.48	3.41	7.20
3α,11β-diOHDHP4	<LOQ	1.66	1.82	1.53	<LOQ	4.18	5.94	7.00	<LOQ	3.17	2.53	4.32	<LOQ	5.84	4.54	8.90
Corticosterone	5.96	1.99	1.79	1.90	6.65	5.29	7.37	6.22	7.37	1.53	2.88	2.17	9.08	5.01	8.40	5.89
11OHDHT	2.54	2.76	1.18	0.45	10.63	5.76	9.84	5.72	2.55	3.38	3.11	0.38	8.14	3.49	4.88	3.64
Pregnanetriol	1.49	0.88	1.09	0.91	7.05	6.46	6.18	9.04	1.77	0.78	0.69	0.58	5.06	3.85	1.50	1.50
Aldosterone	11.02	4.49	4.98	3.67	12.95	5.20	4.33	9.74	14.40	4.97	4.18	3.78	11.79	2.96	1.71	8.86
21-dF	12.77	1.52	0.79	5.02	11.47	7.57	3.78	3.26	8.64	2.35	2.93	1.19	13.72	4.83	2.79	4.60
E	3.44	0.57	3.09	1.28	7.69	5.23	4.65	5.11	2.96	1.58	2.82	2.74	6.58	2.50	5.57	2.92
11K3αDIOL	4.07	4.88	2.52	0.97	13.44	8.97	6.00	4.98	6.52	2.96	2.52	1.31	4.64	1.99	6.40	4.92
11KPdiol	5.96	0.78	1.08	1.93	13.82	11.75	6.21	8.85	8.60	2.05	1.59	1.07	6.71	7.88	8.22	8.39
11OHT	1.07	0.85	1.45	0.81	4.71	3.59	6.02	4.76	1.09	0.83	1.04	0.67	5.87	4.28	4.24	2.74
Prednisone	8.47	6.92	-	4.53	6.45	6.04	-	7.70	9.87	6.83	-	3.02	9.32	7.25	-	5.86
11OHPdiol	<LOQ	3.85	4.42	2.30	<LOQ	10.32	8.48	6.36	<LOQ	6.70	3.66	1.91	<LOQ	9.25	4.35	4.69
11OH3αDIOL	<LOQ	2.50	1.35	3.25	<LOQ	13.66	13.63	6.34	<LOQ	0.91	1.26	1.41	<LOQ	8.40	3.63	7.01
F	11.13	3.75	2.05	2.90	5.36	5.13	3.81	2.63	10.28	7.49	3.82	2.41	6.68	0.98	3.53	4.43
18OHcorticosterone	12.23	4.73	4.48	5.22	12.50	10.87	9.30	6.88	11.91	6.29	7.38	7.29	12.78	3.60	3.90	6.09

Deuterium-labelled internal reference standards	**Accuracy (%RSD)** **50 µL mix (ng per individual steroid)**	**Precision (%RSD)** **50 µL mix (ng per individual steroid)**
DHP4-d6	11.52	10.07
P4-d9	7.98	7.74
A4-d7	8.05	3.86
AST-d2	7.88	10.34
DHT-d4	11.74	8.26
DHEA-d6	3.55	4.53
17OHP4-d8	5.08	4.79
T-d2	6.66	4.87
11Ketiocholanolone-d5	7.85	3.10
11OHA4-d7	7.07	3.78
11KDHT-d3	10.61	7.29
3αDIOL-d3	7.27	8.09
11KT-d3	11.13	6.74

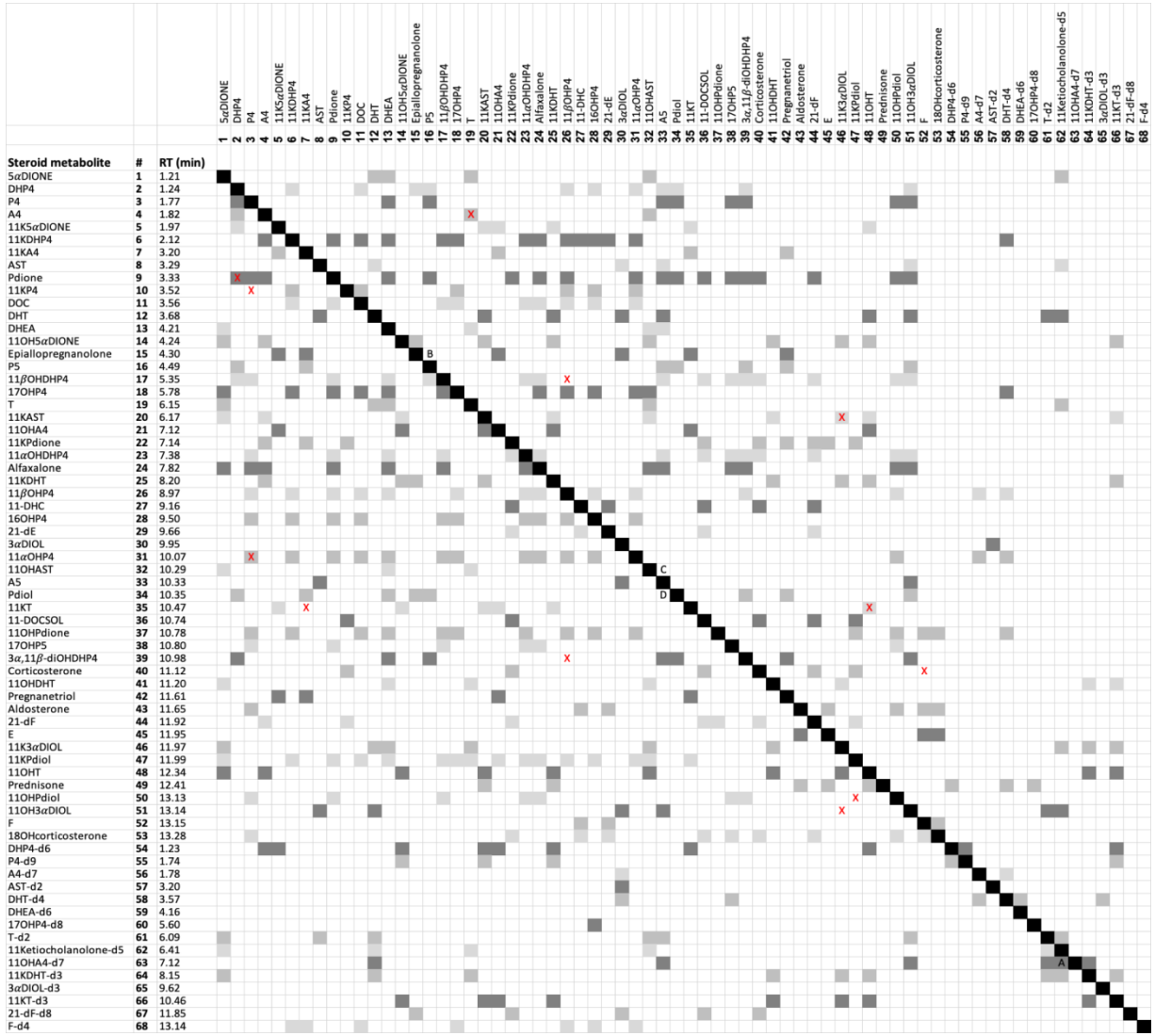
21dF-d8	8.53				5.48											
F-d4	9.57				4.19											
QC compounds	**Accuracy (%RSD)**				**Precision (%RSD)**											
					Day 1				**Day 2**				**Day 3**			
	ng/mL				**ng/mL**											
	3.75	**37.5**	**75**	**375**	**3.75**	**37.5**	**75**	**375**	**3.75**	**37.5**	**75**	**375**	**3.75**	**37.5**	**75**	**375**
(+/-) Trans-stilbene oxide	<LOQ	4.15	6.53	7.03	<LOQ	5.45	9.21	8.93	<LOQ	10.64	5.10	4.91	<LOQ	10.51	3.35	5.14
Thymine	<LOQ	7.38	5.54	3.08	<LOQ	6.66	4.97	4.40	<LOQ	9.24	2.62	2.98	<LOQ	9.09	0.84	1.93
Sulfamethoxazole	3.58	2.04	2.72	2.47	6.36	4.20	2.76	1.60	9.64	5.04	3.04	1.70	10.27	4.04	1.58	1.29
Sulfamethizole	8.56	5.08	3.77	5.14	16.47	6.60	4.96	10.54	15.75	5.25	3.14	2.32	10.08	4.25	2.48	4.10

Table 6 Method validation data: Recovery, matrix effect and process efficiency, n=8, calculated from response values for steroid metabolites.

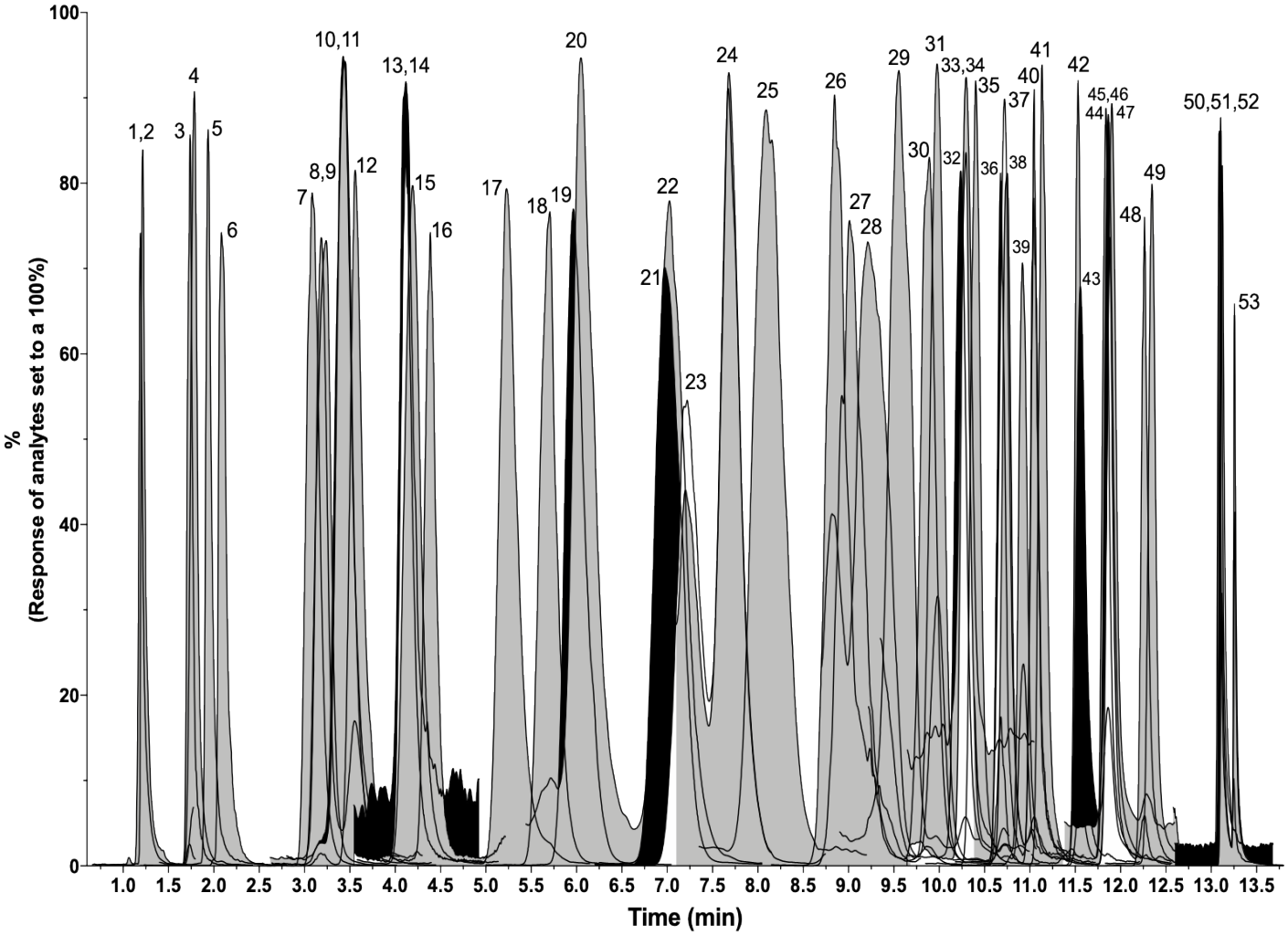
Steroid metabolite	Recovery (%)				Matrix effect (%)				Process efficiency (%)			
	ng/mL (pg on the column)				ng/mL (pg on the column)				ng/mL (pg on the column)			
	2 (4)	20 (40)	100 (200)	200 (400)	2 (4)	20 (40)	100 (200)	200 (400)	2 (4)	20 (40)	100 (200)	200 (400)
5αDIONE	110.00	107.91	102.13	102.99	-1.17	-1.59	-2.35	1.13	107.18	106.05	99.29	103.28
DHP4	107.07	92.73	99.82	107.83	-4.64	13.74	-3.40	0.92	100.68	105.27	96.64	108.57
P4	101.12	104.77	97.17	97.35	-6.59	1.18	-9.44	3.30	94.53	104.74	87.50	98.90
A4	104.71	94.90	94.42	95.68	1.98	5.69	5.93	5.54	103.55	99.85	100.38	101.86
11K5αDIONE	98.71	100.82	100.17	96.41	6.28	10.78	12.02	3.00	102.73	109.41	113.21	98.58
11KDHP4	101.66	95.60	86.47	85.28	7.69	-11.38	-5.27	13.34	108.33	84.10	80.58	95.76
11KA4	105.78	106.33	105.04	98.50	-9.94	-7.33	0.68	3.00	95.54	99.68	105.81	101.10
AST	<LOQ	104.29	97.83	95.84	<LOQ	8.27	1.86	0.57	<LOQ	111.85	99.20	95.96
Pdione	<LOQ	<LOQ	108.74	100.81	<LOQ	<LOQ	-12.86	-0.27	<LOQ	<LOQ	93.91	100.23
11KP4	105.97	112.90	106.64	101.92	3.56	-4.67	-0.32	0.75	109.08	107.42	105.85	102.72
DOC	<LOQ	107.91	109.97	104.61	<LOQ	-0.81	2.00	9.22	<LOQ	106.54	111.91	114.19
DHT	105.73	104.06	104.86	99.44	2.06	0.15	-3.31	7.09	107.81	103.02	101.37	106.34
11OH5αDIONE	<LOQ	96.92	103.10	104.40	<LOQ	-2.24	8.83	10.68	<LOQ	94.73	112.10	115.69
DHEA	<LOQ	95.60	94.92	97.61	<LOQ	-2.67	2.06	8.28	<LOQ	92.12	96.65	105.37
Epiallopregnanolone	104.68	90.97	90.35	91.84	-8.17	0.38	3.82	4.41	96.77	91.17	93.77	95.80
P5	<LOQ	103.46	86.91	90.25	<LOQ	-2.58	2.82	4.33	<LOQ	100.32	89.38	93.73
11βOHDHP4	<LOQ	99.02	106.86	93.66	<LOQ	8.86	7.06	12.83	<LOQ	107.44	114.01	105.57
17OHP4	100.94	97.40	99.85	97.85	-2.98	-6.48	0.77	3.69	97.58	90.67	100.62	101.36
T	107.17	101.07	101.35	99.36	-4.18	-2.53	-1.08	4.94	102.70	101.07	100.28	104.21
11KAST	<LOQ	107.81	107.56	99.65	<LOQ	-9.66	-10.46	-0.95	<LOQ	96.05	95.42	98.74
11OHA4	108.86	107.63	104.86	104.07	2.67	-2.92	-3.06	6.10	110.71	104.08	101.47	110.35
11KPdione	<LOQ	106.65	104.79	103.53	<LOQ	-1.00	6.49	9.34	<LOQ	104.70	111.50	113.04
11αOHDHP4	<LOQ	107.26	104.18	106.83	<LOQ	-4.11	1.23	1.54	<LOQ	101.96	104.45	107.86
Alfaxalone	100.51	98.59	95.96	95.38	13.40	1.20	-5.70	7.05	112.65	98.56	90.37	101.48
11KDHT	106.29	98.65	100.55	98.74	0.91	-5.11	-3.30	5.10	105.74	93.00	97.19	103.43
11βOHP4	102.91	112.60	103.37	105.68	12.80	-2.44	-8.70	-10.50	116.23	109.01	94.20	94.58
11-DHC	108.68	107.89	107.47	112.99	-0.11	-5.38	-9.00	-12.42	108.26	101.28	97.33	98.78
16OHP4	113.42	113.24	103.74	105.12	1.39	-2.12	-7.67	-7.39	114.71	109.67	95.52	97.50
21-dE	104.88	111.94	109.87	113.02	-8.77	-12.73	-15.24	-11.41	95.35	97.08	92.99	99.55
3αDIOL	<LOQ	92.47	95.38	101.90	<LOQ	8.07	-6.13	-1.62	<LOQ	99.60	89.57	100.03
11αOHP4	109.07	111.24	103.98	107.19	-3.37	-13.51	-8.05	-9.97	104.99	95.92	95.42	96.18
11OHAST	<LOQ	96.75	99.66	104.81	<LOQ	-5.60	-6.19	-3.85	<LOQ	88.86	92.61	100.38
A5	<LOQ	111.68	96.71	94.88	<LOQ	3.16	4.33	12.46	<LOQ	111.46	100.96	106.96
Pdiol	<LOQ	89.82	92.20	92.76	<LOQ	9.14	8.21	4.87	<LOQ	97.51	99.47	97.00
11KT	91.04	99.64	101.74	101.17	12.79	1.31	1.60	14.25	102.18	101.31	103.76	101.17

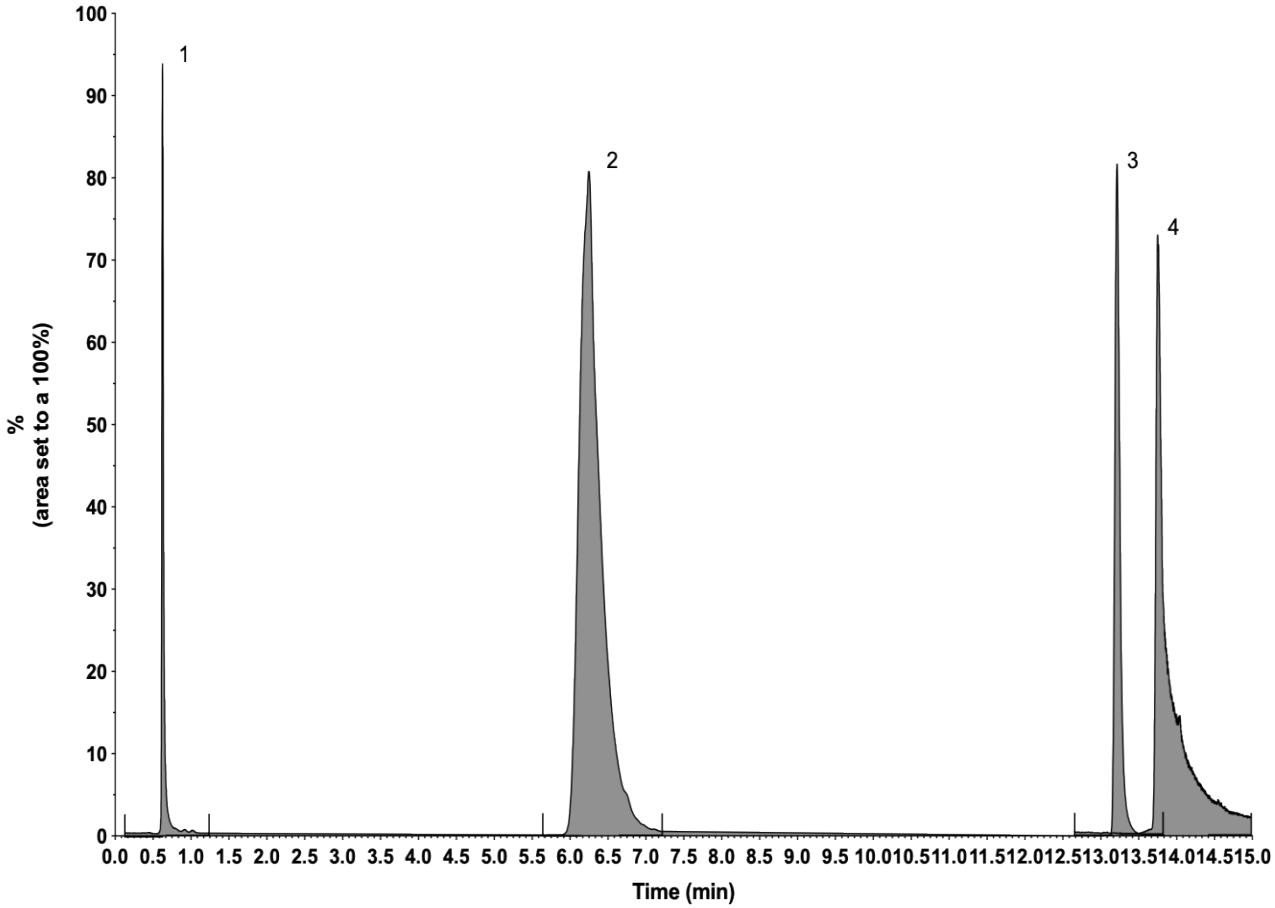
11-DOCSOL	111.44	101.26	102.29	98.05	-1.83	-2.15	2.33	12.07	96.87	101.26	104.65	109.90
11OHPdione	<LOQ	109.23	98.83	104.78	<LOQ	0.48	10.82	4.36	<LOQ	105.09	107.56	109.15
17OHP5	<LOQ	100.55	90.35	95.88	<LOQ	7.52	2.04	-3.38	<LOQ	100.55	91.87	92.62
3α,11β-diOHDHP4	<LOQ	103.17	96.45	96.93	<LOQ	14.24	5.07	4.15	<LOQ	108.24	101.35	100.91
Corticosterone	107.66	106.34	106.21	112.53	-5.30	-2.42	-1.16	-9.15	101.37	102.79	104.85	101.18
11OHDHT	100.94	108.89	111.00	111.51	2.54	0.20	-9.16	-1.31	102.32	107.55	100.68	109.72
Pregnanetriol	108.51	96.67	99.87	105.72	-1.29	3.48	-1.10	-1.52	102.07	100.15	97.94	103.56
Aldosterone	108.21	106.94	97.59	97.91	-8.70	-3.64	-5.09	7.22	96.61	101.23	97.59	105.03
21-dF	107.55	111.04	109.92	111.70	-0.31	1.80	-2.65	-1.46	102.18	112.53	106.67	109.98
E	99.17	111.51	110.58	100.51	-1.12	-0.73	-6.77	5.26	96.90	108.82	102.68	105.62
11K3αDIOL	115.95	106.18	105.80	109.33	-3.12	5.26	-4.70	-0.84	111.03	111.13	100.33	108.09
11KPdiol	97.52	111.61	112.20	108.79	-5.47	3.35	-3.30	4.28	88.48	113.86	108.12	112.99
11OHT	109.89	111.94	106.87	109.23	-1.84	-1.35	-3.10	-2.17	107.60	109.37	103.41	106.74
Prednisone	96.86	97.13	-	97.25	-11.17	9.40	-	9.75	86.04	106.27	-	106.73
11OHPdiol	<LOQ	87.58	101.07	92.16	<LOQ	2.91	1.67	8.57	<LOQ	88.68	101.50	100.17
11OH3αDIOL	<LOQ	100.09	98.94	101.30	<LOQ	11.35	-11.45	4.91	<LOQ	107.22	86.48	106.25
F	101.59	100.30	103.31	99.57	9.69	-3.20	-5.71	3.61	111.08	96.52	97.50	103.14
18OHcorticosterone	100.93	100.19	98.42	91.27	-9.73	-8.46	-7.67	7.24	89.50	91.03	89.63	97.45

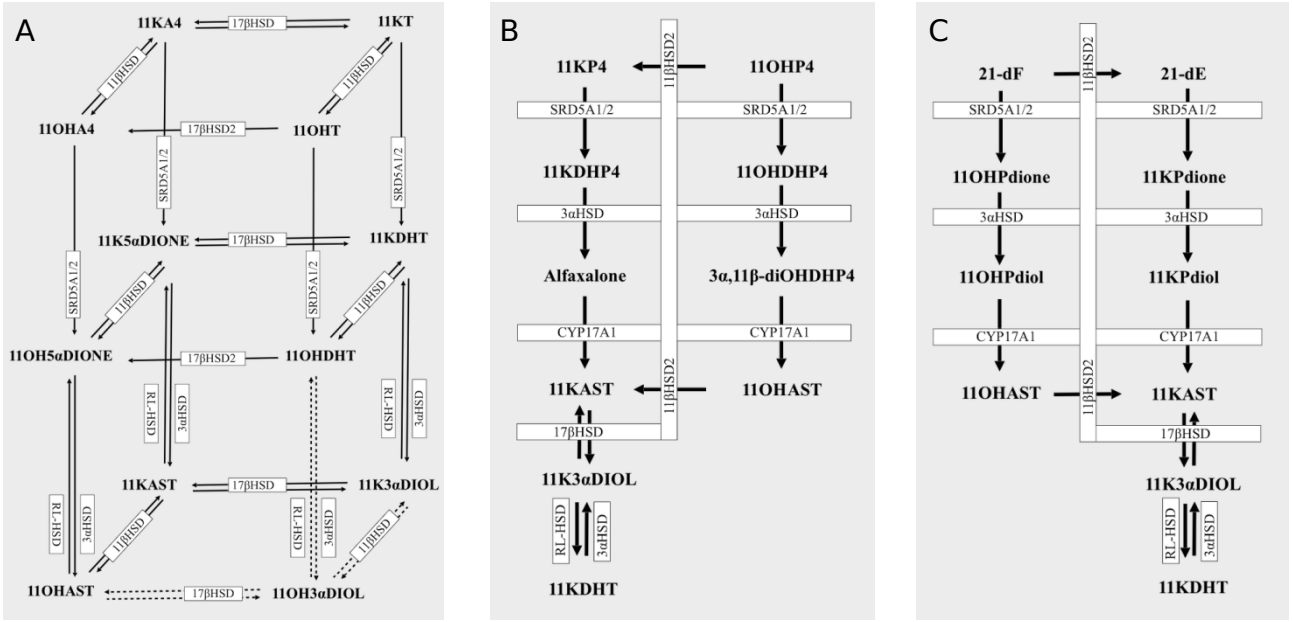
List of figures



Steroid metabolite	#	RT (min)
5αDIONE	1	1.21
DHP4	2	1.24
P4	3	1.77
A4	4	1.82
11K5αDIONE	5	1.97
11KDHP4	6	2.12
11KA4	7	3.20
AST	8	3.29
Pdione	9	3.33
11KP4	10	3.52
DOC	11	3.56
DHT	12	3.68
DHEA	13	4.21
11OH5αDIONE	14	4.24
Epiallopregnanolone	15	4.30
P5	16	4.49
11βOHDHP4	17	5.35
17OHP4	18	5.78
T	19	6.15
11KAST	20	6.17
11OHA4	21	7.12
11KPdione	22	7.14
11αOHDHP4	23	7.38
Alfaxalone	24	7.82
11KDHT	25	8.20
11βOHP4	26	8.97
11-DHC	27	9.16
16OHP4	28	9.50
21-dE	29	9.66
3αDIOL	30	9.95
11αOHP4	31	10.07
11OHAST	32	10.29
A5	33	10.33
Pdiol	34	10.35
11KT	35	10.47
11-DOCSOL	36	10.74
11OHPdione	37	10.78
17OHP5	38	10.80
3α,11β-diOHDHP4	39	10.98
Corticosterone	40	11.12
11OHDHT	41	11.20
Pregnanetriol	42	11.61
Aldosterone	43	11.65
21-dF	44	11.92
E	45	11.95
11K3αDIOL	46	11.97
11KPdiol	47	11.99
11OHT	48	12.34
Prednisone	49	12.41
11OHPdiol	50	13.13
11OH3αDIOL	51	13.14
F	52	13.15
18OHcorticosterone	53	13.28
DHP4-d6	54	1.23
P4-d9	55	1.74
A4-d7	56	1.78
AST-d2	57	3.20
DHT-d4	58	3.57
DHEA-d6	59	4.16
17OHP4-d8	60	5.60
T-d2	61	6.09
11Ketiocholanolone-d5	62	6.41
11OHA4-d7	63	7.12
11KDHT-d3	64	8.15
3αDIOL-d3	65	9.62
11KT-d3	66	10.46
21-dF-d8	67	11.85
F-d4	68	13.14







Supplemental figure 1 Steroidogenic pathways depicting the (A) 11OHA4- and (B, C) C11-oxy backdoor pathways, leading to the production of C11-oxy steroid intermediate metabolites and end-products. Solid arrows, confirmed reactions; dashed arrows, unconfirmed *in vitro* reactions.

Supplemental table 1 Ion ratios of the steroid metabolites.

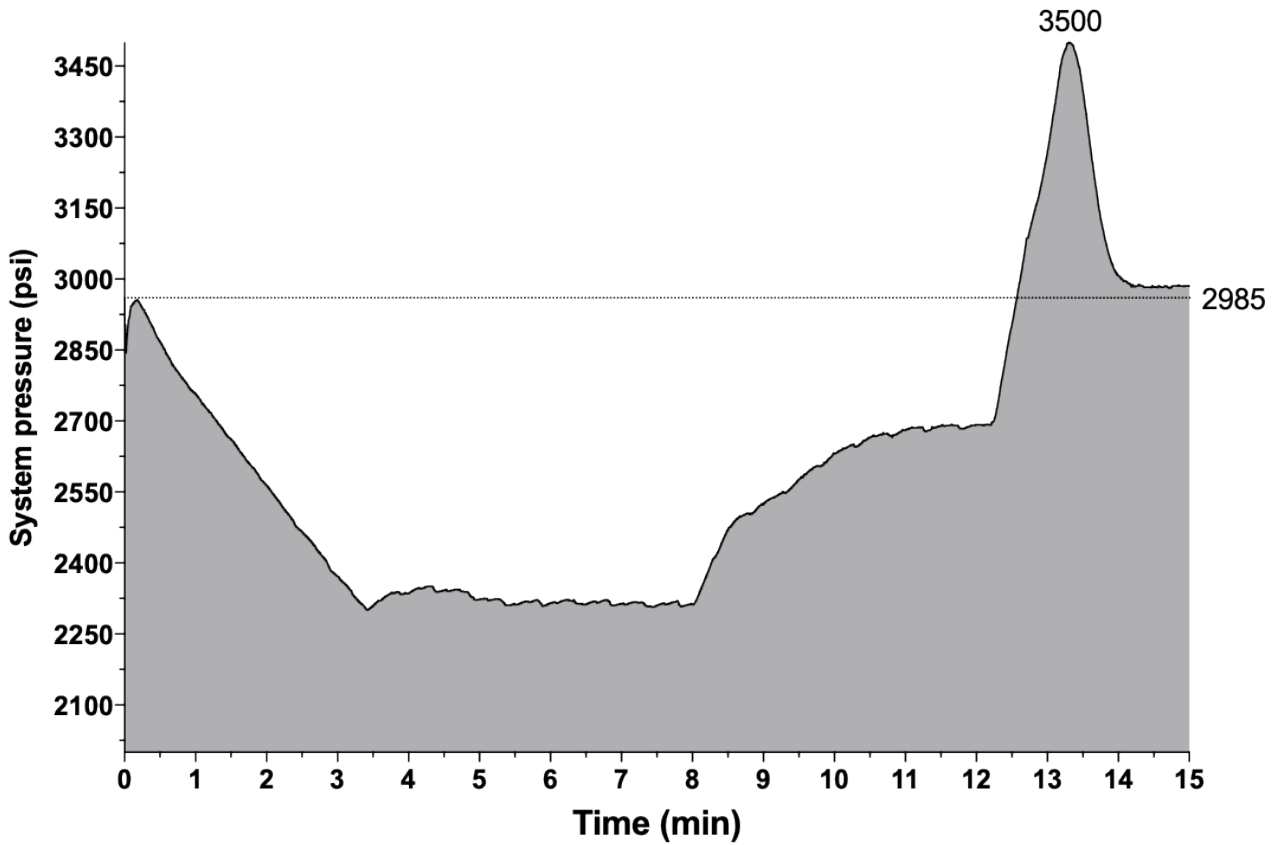
Steroid metabolites	Molecular ion species (QN > QL)	Ion ratio (QL/QN)	%RSD	n
5αDIONE	253.1 > 271.2	1.21	11.76	7
DHP4	95.0 > 105.2	1.08	13.56	8
P4	79.15 > 297.0	0.71	9.94	10
A4	123.0 > 173.0	0.56	14.02	7
11K5αDIONE	241.0 > 248.9	0.97	10.88	7
11KDHP4	277.3 > 255.0	0.68	5.89	7
11KA4	121.1 > 265.21	0.82	2.13	7
AST	185.1 > 69.07	0.31	14.51	5
Pdione	228.76 > 70.82	1.09	10.58	4
11KP4	121.0 > 285.11	0.37	4.59	8
DOC	117.02 > 130.89	1.50	14.00	7
DHT	255.29 > 104.9	0.21	13.79	8
11OH5αDIONE	269.2 > 211.0	0.47	6.09	6
DHEA	253.2 > 213.0	0.46	6.94	6
Epiallopregnanolone	189.2 > 283.15	0.80	14.49	8
P5	80.97 > 131.0	0.75	2.90	5
11βOHDHP4	85.0 > 159.0	0.66	13.85	6
17OHP4	312.8 > 295.0	0.70	4.50	7
T	123.0 > 271.18	0.51	8.66	9
11KAST	229.1 > 147.2	0.67	13.64	7
11OHA4	267.2 > 285.2	0.97	3.65	7
11KPdione	329.0 > 311.1	0.76	4.56	6
11αOHDHP4	297.3 > 85.2	0.55	15.09	6
Alfaxalone	297.0 > 85.0	0.99	4.97	7
11KDHT	251.0 > 144.9	0.62	14.56	7
11βOHP4	295.01 > 313.0	1.42	10.29	8
11-DHC	121.15 > 163.09	0.18	11.50	7
16OHP4	97.01 > 109.1	0.69	10.45	10
21-dE	163.2 > 121.08	0.50	13.53	9
3αDIOL	257.25 > 175.0	0.27	12.07	5
11αOHP4	295.2 > 313.05	0.97	11.40	9

11OHAST	271.3 > 253.2	0.74	9.53	5
A5	201.17 > 185.28	0.70	15.11	6
Pdiol	299.0 > 111.0	0.68	12.56	7
11KT	147.1 > 285.0	0.91	14.72	9
11-DOCSOL	123.04 > 78.94	0.95	12.48	10
11OHPdione	114.99 > 183.25	0.70	14.72	5
17OHP5	159.12 > 133.1	0.77	6.79	7
3α,11β-diOHDHP4	159.0 > 95.0	0.98	12.06	6
Corticosterone	97.1 > 104.98	0.97	4.50	8
11OHDHT	271.0 > 213.2	0.59	14.29	7
Pregnanetriol	283.2 > 187.0	0.29	13.39	7
Aldosterone	343.0 > 315.0	0.58	5.14	7
21-dF	95.39 > 210.9	1.42	12.80	7
E	163.16 > 120.94	0.27	10.60	10
11K3αDIOL	271.0 > 253.0	0.55	14.53	9
11KPdiol	289.24 > 271.32	0.96	11.41	9
11OHT	269.0 > 121.0	0.92	4.12	10
Prednisone	147.0>171.0	0.77	2.36	9
11OHPdiol	279.1 > 145.1	0.62	13.40	7
11OH3αDIOL	107.0 > 147.2	1.73	8.80	5
F	121.08 > 308.98	0.15	14.79	12
18OHcorticosterone	269.2 > 147.0	0.58	12.73	9
(+/-) Trans-stilbene oxide	105.12 > 76.99	0.39	11.84	6
Thymine	53.97 > 55.95	0.58	11.00	5
Sulfamethoxazole	108.06 > 92.17	1.35	9.77	9
Sulfamethizole	107.99 > 92.17	1.19	5.99	7

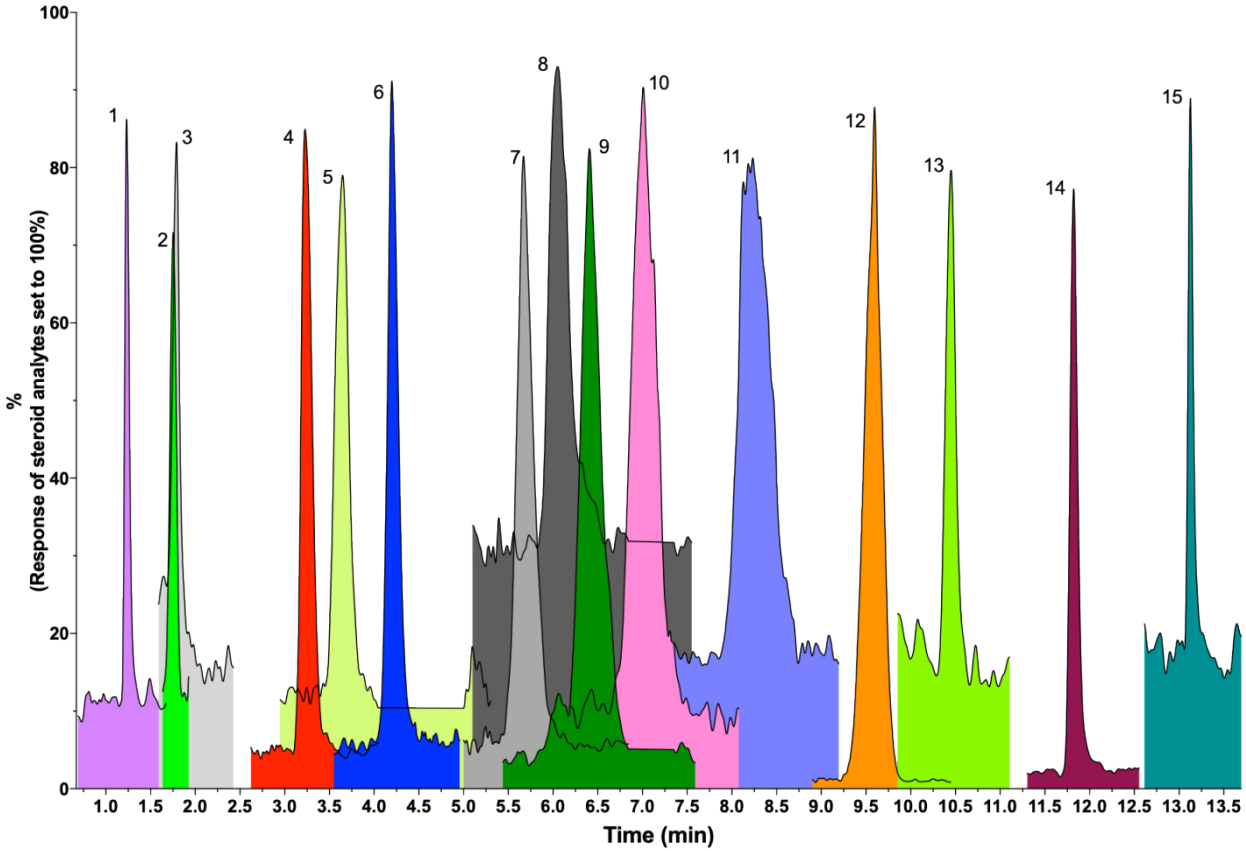
Supplemental table 2 Calculated response factors (Rf) in the absolute quantification of steroid reference standards contaminated with precursor steroids.

Steroid metabolites	Rf (in a serum matrix)
F	0.005
11βOHP4	0.034
11OHT	0.014
11KA4	0.049
DHP4	0.123
11KPdiol	0.052
11K3αDIOL	0.040
P4	0.029
T	0.107

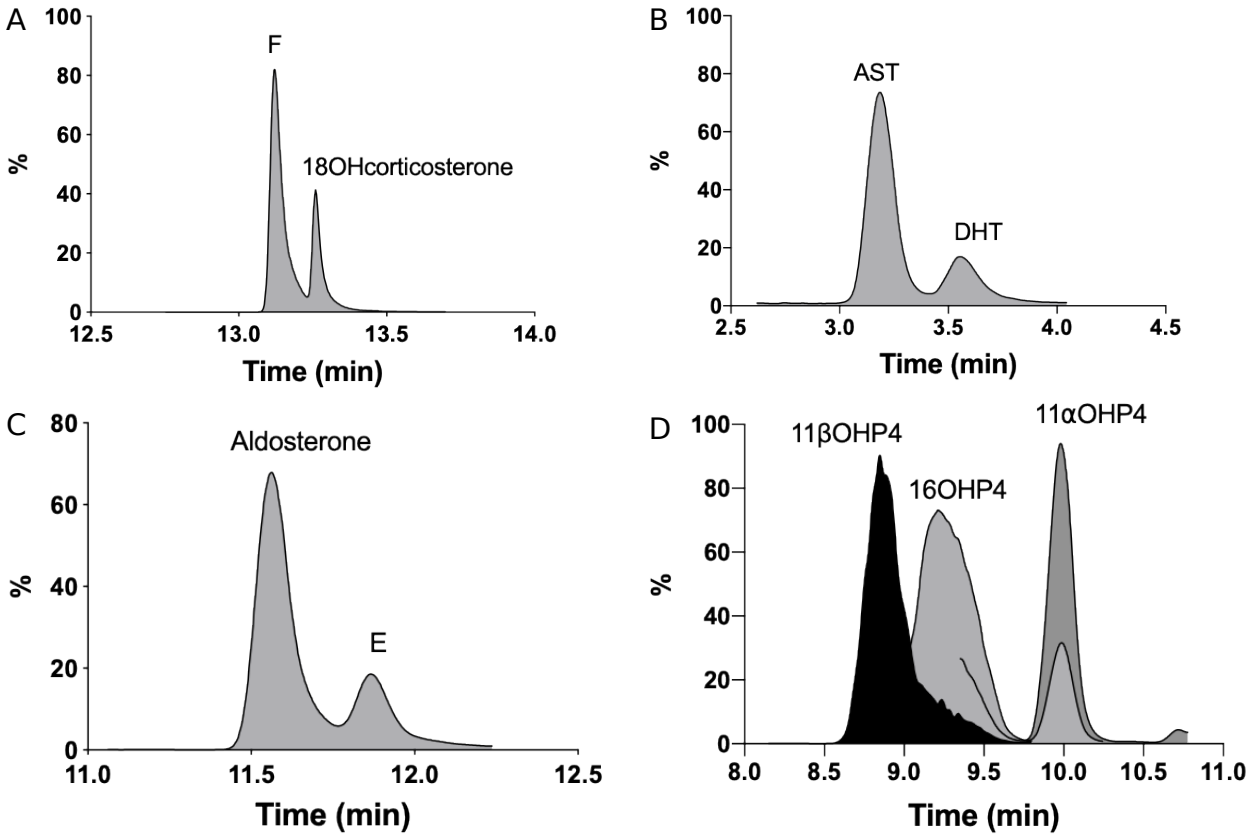
*The response of each of these steroids, at the concentrations in the standard curve, should be multiplied by the given Rf, and subtracted from the original response values to generate new standard curves.



Supplemental figure 2 System pressure from the start to the completion of the run.



Supplemental figure 3 UPC^2-MS/MS separation of 15 reference deuterium-labelled standards. Internal standards (ng per sample to be analysed), are depicted in the elution order; (1) DHP4-d6 (5 ng), 1.23 min; (2) P4-d9 (10 ng), 1.74 min; (3) A4-d7 (10 ng), 1.78 min; (4) AST-d2 (15 ng), 3.2 min; (5) DHT-d4 (5 ng), 3.57 min; (6) DHEA-d6 (25 ng), 4.16 min; (7) 17OHP4-d8 (10 ng), 5.6 min; (8) T-d2 (10 ng), 6.09 min; (9) 11Ketiocholanolone-d5 (20 ng), 6.41 min; (10) 11OHA4-d7 (1.5 ng), 7.12 min; (11) 11KDHT-d3 (5 ng), 8.15 min; (12) 3αDIOL-d3 (25 ng), 9.62 min; (13) 11KT-d3 (5 ng), 10.46 min; (14) 21-dF-d8 (1 ng), 11.85 min; and (15) F-d4 (5 ng), 13.14 min.



Supplemental figure 4 Examples of the UPC^2-MS/MS separation of (A) F and 18OHcorticosterone in F's channel, (B) AST and DHT in AST's channel, (C) aldosterone and E in aldosterone's channel and (D) 11βOHP4, 16OHP4 and 11αOHP4 in 11βOHP4's channel —showing the presence of multiple steroids in single MRMs.

Supplemental table 3 Method validation data of the dilution protocol for steroid metabolites diluted 10-fold. Precision and recovery, n=6, except where indicated, calculated from response values at 200 ng/mL.

Steroid metabolite	Precision (%RSD)	Recovery (%)*
5αDIONE	5.73	99.79
DHP4	5.34	98.35
P4	13.34	88.83
A4 (n=5)	9.12	92.68
11K5αDIONE (n=5)	12.47	99.56
11KDHP4 (n=5)	10.59	91.58
11KA4 (n=5)	10.03	95.51
AST	8.92	93.92
Pdione	2.81	125.24
11KP4 (n=5)	8.28	92.01
DOC	5.26	71.13
DHT (n=5)	5.93	66.92
11OH5αDIONE	8.19	73.16
DHEA	4.93	81.60
Epiallopregnanolone	14.52	88.80
P5	6.62	97.93
11βOHDHP4	2.89	96.64
17OHP4	14.80	87.61
T	10.50	73.65
11KAST (n=5)	7.93	102.13
11OHA4	10.59	84.28
11KPdione	3.97	98.23
11αOHDHP4 (n=4)	10.75	80.36
Alfaxalone (n=5)	12.62	95.55
11KDHT	6.77	93.96
11βOHP4 (n=5)	8.19	79.23
11-DHC	5.73	83.30
16OHP4	11.35	84.25
21-dE	6.92	84.28
3αDIOL (n=5)	10.50	100.27

11αOHP4	9.37	96.51
11OHAST (n=5)	11.08	144.13
A5	11.61	152.75
Pdiol	6.65	115.89
11KT (n=5)	6.13	89.94
11-DOCSOL	10.22	90.14
11OHPdione	10.26	97.00
17OHP5 (n=5)	10.39	121.07
3α,11β-diOHDHP4	5.56	95.00
Corticosterone	6.05	85.32
11OHDHT	12.45	87.64
Pregnanetriol	8.35	91.12
Aldosterone	5.84	87.82
21-dF	5.70	96.59
E	7.29	91.29
11K3αDIOL	8.31	82.88
11KPdiol	8.22	88.47
11OHT	8.89	86.30
Prednisone	4.92	94.73
11OHPdiol (n=5)	14.85	95.17
11OH3αDIOL	5.33	81.21
F	12.11	84.53
18OHcorticosterone	13.74	87.27

*The dilution protocol was validated by diluting the 200 ng/mL precision samples 10-fold with the serum matrix and the resulting diluted response values were compared with the undiluted precision samples and the percentage recoveries calculated (2nd column).
A 10-fold dilution resulted in the loss of AST-d2, DHEA-d6 and 11KT-d3 as internal standards (see supplemental Table 4), therefore certain steroid areas were divided by other internal standards as follows: 11KA4/A4-d7; AST/DHT-d4; 11KP4/A4-d7; DHEA/DHT-d4; epiallopregnanolone/17OHP4-d8; 11OH5αDIONE/DHT-d4; P5/DHT-d4; 11OHAST/3αDIOL-d3; A5/3αDIOL-d3; 11KT/3αDIOL-d3; Pdiol/3αDIOL-d3; 11-DOCSOL/21-dF-d8; 11OHPdione/21-dF-d8; 17OHP5/21-dF-d8; 3α,11β-diOHDHP4/21-dF-d8 and corticosterone/21-dF-d8.

Supplemental table 4 Method validation data of the dilution protocol for the internal standards. Precision (n=4) were calculated from area values from 50 µL of the internal standard mix (ng per individual deuterated steroid as listed in the manuscript).

Deuterium-labelled internal reference standards	**Precision (%RSD)**	
	3-fold	**10-fold**
DHP4-d6	4.38	6.13
P4-d9	9.39	9.59
A4-d7	6.51	7.91
AST-d2	10.29	<LOQ
DHT-d4	2.14	7.21
DHEA-d6	10.75	<LOQ
17OHP4-d8	2.25	5.82
T-d2	3.74	7.31
11Ketiocholanolone-d5	6.58	9.17
11OHA4-d7	3.76	10.85
11KDHT-d3	1.83	8.70
3αDIOL-d3	8.65	12.48
11KT-d3	8.32	<LOQ
21dF-d8	3.44	5.27
F-d4	4.40	12.22

Supplemental table 5 Method validation data of the dilution protocol for the QC samples diluted 10-fold. Accuracy, precision and recovery, n=8, were calculated from area values at 37.5 ng/mL.

QC compounds	Accuracy (%RSD)	Precision (%RSD) Day 1	Recovery (%)*	Precision (%RSD) Day 2	Recovery (%)*	Precision (%RSD) Day 3	Recovery (%)*
(+/-) Trans-stilbene oxide	5.42	6.03	100.08	6.38	118.89	8.56	95.09
Thymine	4.57	6.37	100.79	6.43	98.40	6.99	96.17
Sulfamethoxazole	3.95	4.00	91.81	3.00	96.93	2.61	98.10
Sulfamethizole	3.87	8.85	100.00	3.48	109.92	2.99	99.88

The dilution protocol was validated by diluting the 375 ng/mL accuracy and precision samples 10-fold with the serum matrix resulting in the concentration at 37.5 ng/mL reported in the table.

*The resulting diluted peak areas of the precision samples were compared with the 37.5 ng/mL precision samples and the recoveries (2nd column of precision) calculated.

Supplemental table 6 Method validation data: Accuracy bias determined for the steroid metabolites.

	Steroid metabolites	Accuracy bias (%RSD) (n=8), except for T (n=6)	% recovery (n=6), except for 11-DOCSOL and T (n=4); and 21-dF and DOC (n=5)
Mix 1	17OHP4	7.35	97.05
	A4	7.55	75.78
	11-DOCSOL	5.10	101.59
Mix 2	F	3.80	99.01
	21-dF	7.53	102.82
	DOC	8.12	72.05
	T	7.07	76.96

Declaration by the candidate:

With regard to Addendum A the nature and scope of my contribution were as follows:

Nature of contribution: Desmaré van Rooyen completed the following: developed and validated the UPC^2-MS/MS method with Therina du Toit which entailed characterisation of the steroid compounds and control references, determining crosstalk, separation of steroids, steroid standards and reference compounds; contributed to the compilation of Figure 1; planned and discussed the first draft of the manuscript.

Extent of contribution: method development and validation, data collection and processing 50 %; manuscript 10 %

The following co-authors have contributed to the manuscript:
Name; e-mail address; nature of contribution; extent of contribution (%)

- Therina du Toit: tdutoit@sun.ac.za; responsible for method development and validation, data collection and processing 50 %, compiled the first draft, figures and tables, supplementary file, edited and completed the final manuscript (70 %) with AC Swart (20 %).
- Maria A Stander: lcms@sun.ac.za; read and edited the final manuscript draft.
- Stephen L. Atkin: satkin@rcsi-mub.com; read and edited the final manuscript draft.
- Amanda C. Swart; acswart@sun.ac.za; principal investigator, corresponding author, editing of the manuscript.

Signature of candidate: *_____

Date: 16/03/2020

Declaration by co-authors:

The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to the manuscript (Addendum A),
2. no other authors contributed to the the manuscript besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in the addendum of this dissertation.

Signature	Institutional affiliation	Date
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*_____	RCSI Bahrain, Manama	16 March 2020
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* Declaration with signature in possession of candidate and supervisor.