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Contributions of cortisol and corticosterone to metabolic regulation in humans

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Declaration

I hereby declare that this thesis was written by me, and that the data published in this thesis are the result of my own work.

The work in this thesis has not previously been submitted for any other degree or qualification.

Catriona Kyle

Edinburgh, January 2018

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Contents

Abstract

Both cortisol and corticosterone circulate in human plasma however corticosterone has been relatively neglected in human research to date. There is evidence of distinct regulation within different tissues with the transmembrane transporter ABCB1, highly expressed in the brain, exporting cortisol but not corticosterone. This may account for the relative accumulation of corticosterone in the CNS. In contrast, ABCC1, highly expressed in adipose tissue and skeletal muscle, exports corticosterone but not cortisol, suggesting cortisol is the principal glucocorticoid acting in these tissues.

We tested the hypotheses that: (i) corticosterone physiology in humans is different to that of cortisol; (ii) inhibition of ABCC1 increases binding of corticosterone to corticosteroid receptors in adipose tissue and skeletal muscle but has no central CNS effect; and (iii) corticosterone is superior to cortisol as a basis for glucocorticoid replacement therapy with fewer metabolic side effects.

We compared paired salivary and plasma samples from 10 healthy individuals. Plasma corticosterone showed a similar diurnal variation to cortisol but salivary corticosterone was low and did not correlate with plasma concentrations.

A placebo-controlled randomised crossover study was carried out in 14 healthy individuals comparing receptor occupancy of glucocorticoids centrally and peripherally with and without ABCC1 inhibition. Receptor occupancy was assessed through displacement with MR and GR antagonists potassium canrenoate and mifepristone. Centrally, ABCC1 inhibition caused increased activation of the HPA

axis after MR and GR antagonism. Peripherally, we were unable to show displacement from adipose tissue or skeletal muscle.

A further placebo-controlled randomised crossover study is still ongoing in 16 patients with congenital adrenal hyperplasia, comparing metabolic effects of placebo, cortisol and corticosterone infusions over 6 hours. We present interim data for n=8. ACTH and 17-OHP were suppressed with corticosterone. Metabolic parameters were similar between placebo, cortisol and corticosterone phases.

These data suggest corticosterone physiology is distinct compared with cortisol in humans. We have shown ABCC1 inhibition alters the HPA axis after receptor antagonism which suggests ABCC1 may play more of a key role centrally than previously thought. Corticosterone suppresses ACTH and 17-OHP in the short term in congenital adrenal hyperplasia, highlighting the possibility of its use as an alternative glucocorticoid replacement therapy in the future.

Lay abstract

Natural steroids, called glucocorticoids, are produced by the adrenal glands and play a major role in the response to stress, fighting infection and inflammation and controlling metabolism. Synthetic glucocorticoids are prescribed by doctors for the treatment of numerous conditions such as allergic skin disease and asthma. They are also given to people whose adrenal glands are unable to make sufficient natural glucocorticoids. Although taking glucocorticoids is very effective, side effects are numerous, including weight gain, increased blood pressure and high cholesterol.

There are two main glucocorticoids in humans, cortisol and corticosterone. Cortisol circulates at higher levels than corticosterone in blood and has therefore been regarded as more important. To date, research in humans has focussed on cortisol and most of the synthetic glucocorticoids given to patients are similar to cortisol. We know much less about corticosterone in health and disease but evidence is mounting that cortisol and corticosterone differ in certain important aspects. For example, corticosterone is present at relatively higher levels in the brain and lower levels in fat tissue than in blood. In contrast, cortisol is present at relatively higher levels in fat tissue and lower levels in brain. This is because in the brain, a pump (named ABCB1) removes only cortisol from cells whereas in fat tissue, a pump (named ABCC1) removes only corticosterone. Given that we think a lot of the side effects of glucocorticoids are due to their effects on fat tissue, we wished to investigate whether corticosterone may be a better basis for synthetic glucocorticoid treatment.

First, we compared corticosterone and cortisol levels in blood, saliva and hair of healthy individuals. We then investigated whether blocking the corticosterone pumps in fat tissue changed the body's response to glucocorticoids. Finally, we investigated the potential to use corticosterone as a treatment for patients who cannot produce glucocorticoids naturally by comparing effects with cortisol.

We found that corticosterone levels did not simply mimic cortisol, highlighting it is a distinct glucocorticoid with its own characteristics. The corticosterone pumps play a significant role in glucocorticoid production and response. Corticosterone was comparable to cortisol in replacement therapy over the short term. This highlights the possibility of using corticosterone as an alternative glucocorticoid replacement therapy in the future.

Abstracts from this thesis

Quantitative analysis of an adrenal steroid profile, canrenone and mifepristone in plasma by triple quadrupole mass spectrometry

- Poster presentation at British Endocrinology Society meeting, November 2015

Corticosterone is highly abundant in human saliva and lacks a diurnal rhythm

- Poster presentation at British Endocrinology Society meeting, November 2015

ABCC1 influences negative feedback of the hypothalamic-pituitary-adrenal (HPA) axis in vivo in humans

- Oral presentation at ENDO conference, March 2017
    - Outstanding Abstract Award
- Poster presentation at Scottish Cardiovascular Forum, February 2017

Salivary corticosterone does not reflect plasma concentration in humans

- Poster presentation at UK Clinical Research Facility Network Conference, July 2017

Contributions of cortisol and corticosterone to metabolic regulation in humans

- Oral presentation at Caledonian Society for Endocrinology and Diabetes annual meeting, November 2017

Abbreviations

11β-HSD 1	11β-hydroxysteroid dehydrogenase 1
11β-HSD 2	11β-hydroxysteroid dehydrogenase 2
17-OHP	17-hydroxyprogesterone
A	11-dehydrocorticosterone
ABC	ATP-binding cassette
ABCB1	ABC transporter B1
ABCC1	ABC transporter C1
ACCORD	Academic and Clinical Central Office for Research and Development
ACTH	Adrenocorticotrophic hormone
AI	Augmentation index
AMREC	ACCORD Medical Research Ethics Committee
ANOVA	Analysis of variance
AP	Augmentation pressure
ATBF	Adipose tissue blood flow
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
B	Corticosterone
BBB	Blood brain barrier
BID	Bis in die (twice daily)
BMI	Body mass index
BP	Blood pressure
BSA	Bovine serum albumin
CAH	Congenital adrenal hyperplasia
CaHASE	Congenital Adrenal Hyperplasia Adult Study Executive
cAMP	Cyclic adenosine mono phosphate

CBG	Corticosteroid binding globulin
CNS	Central nervous system
CRH	Corticotropin releasing hormone
CSF	Cerebrospinal fluid
CSHI	Continuous subcutaneous hydrocortisone infusion
CTX	Collagen type 1 cross linked c-telopeptide
CV	Coefficient of variation
CYP	Cytochrome P450
D2-cortisone	1,2-$[^2H]_2$-cortisone
D2-glucose	6,6-$[^2H]_2$-glucose
D4-cortisol	9,11,12,12-$[^2H]_4$-cortisol
D5-glycerol	1,1,2,3,3-$[^2H]_5$-glycerol
D8-corticosterone	2,2,4,6,6,17α,21,21-$[^2H]_8$-corticosterone
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DOC	Deoxycorticosterone
E	Cortisone
EDTA	Ethylenediaminetetraacetate
EHERTS	East Hertfordshire Study
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
ET2DS	Edinburgh Type 2 Diabetes Study
F	Cortisol
FA	Formic acid
FBC	Full blood count
FBF	Forearm blood flow
GC-MS	Gas chromatography- mass spectrometry

gDNA	Genomic DNA
GR	Glucocorticoid receptor
HbA1c	Glycated haemoglobin
HDL	High density lipoprotein
HEK	Human embryonic kidney
HOMA-IR	Homeostasis model assessment- insulin resistance
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
HR	Heart rate
HRP	Horseradish peroxidase
K_d	Dissociation constant
K_i	Substrate inhibition constant
KCan	Potassium Canrenoate
LC-MS/MS	Liquid chromatography- tandem mass spectrometry
LDL	Low density lipoprotein
LFT	Liver function test
LLOQ	Lower limit of quantitation
LOQ	Limit of quantitation
LSD	Least significant difference
m/z	Mass to charge ratio
MC2R	Melanocortin 2 receptor
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NAD	Nicotinamide adenine
NaOH	Sodium hydroxide
NBD	Nucleotide binding domain

NEFA	Non-esterified fatty acids
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
ORCADES	Orkney Complex Disease Study
P1NP	Procollagen type 1 n-propeptide
PKA	Protein kinase A
POMC	Pro-opiomelanocortin
PP	Pulse pressure
PVN	Paraventricular nucleus
PWA	Pulse wave analysis
PWV	Pulse wave velocity
qRT-PCR	Real-time reverse transcription polymerase chain reaction
RIE	Royal Infirmary of Edinburgh
RIECRF	Royal Infirmary of Edinburgh Clinical Research Facility
RSD	Relative standard deviation
RT	Retention time
RU486	Mifepristone
SCN	Suprachiasmatic nucleus
SD	Standard deviation
SEM	Standard error of mean
SLE	Supported liquid extraction
SMR	Standardised mortality ratio
StAR	Steroidogenic acute regulatory protein
T2DM	Type 2 diabetes mellitus
TART	Testicular adrenal rest tumour
TBE	Tris/Borate/EDTA
TFT	Thyroid function test

TMB	Tetramethylbenzidine
TMD	Transmembrane domain
TRPV2	Transient receptor potential vanilloid 2
TTR	Tracer: tracee ratio
UHPLC	Ultra high performance liquid chromatography
UPL	Universal Probe Library
USP	United States Pharmacopeia
WTCRF	Wellcome Trust Clinical Research Facility

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Chapter 1. Introduction

1.1 Glucocorticoid physiology

Glucocorticoids are catabolic steroid hormones secreted by the adrenal cortex with major effects on carbohydrate, protein and lipid metabolism as well as significant anti-inflammatory and immunological actions (Sapolsky et al. 2000). Glucocorticoids play a major role in maintaining homeostasis through energy storage and conservation, immune system modulation and memory formation. They are central to stimulation of cardiovascular responses, promoting energy mobilisation and modulating immune response which are particularly relevant during stress (Vegiopoulos & Herzig 2007; Cain & Cidlowski 2017; de Quervain et al. 2016).

Circulating concentrations of the more abundant glucocorticoid in humans, cortisol, are closely regulated by a negative feedback system with elevations in times of acute stress. This adaptive response allows activation of the afore-mentioned pathways to respond to a stressful stimulus. If elevated circulating cortisol is sustained in the absence of continued stressful stimuli, the response is maladaptive and results in features of Cushing's syndrome such as hypertension and hyperglycaemia. In conditions of impaired glucocorticoid production, synthetic glucocorticoids based on cortisol are administered. Although effective, doses are often supra-physiological and result in similar Cushingoid features. Determining the mechanisms behind maladaptive excess of cortisol and improving synthetic glucocorticoid administration has been a focus of clinical research.

Corticosterone is another, less abundant, human glucocorticoid which has been relatively neglected in the field of human research to date. The extent to which

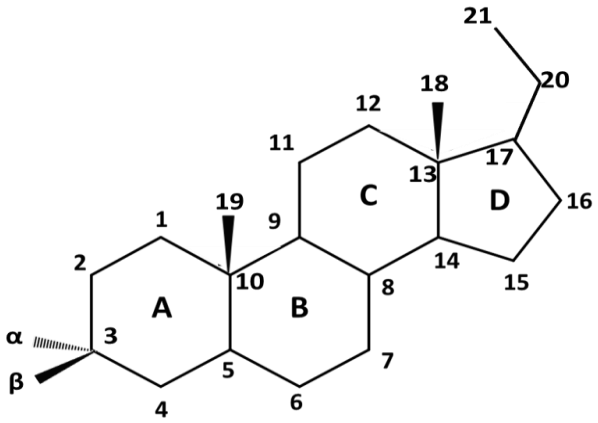
corticosterone mirrors the maladaptive responses of cortisol and the consequences of corticosterone excess are not well defined.

This thesis explores the regulation of cortisol and corticosterone in different tissues and compares their effects in glucocorticoid replacement therapy.

1.1.1 Structure

Glucocorticoids are steroid hormones derived from cholesterol which have a typical molecular configuration. The steroid core structure has seventeen carbon atoms, arranged in four fused rings (named A-D) of which three are cyclohexane and the other cyclopentane (Figure 1-1). The four carbon rings are conventionally labelled by letters and the individual carbon atoms by numbers. Steroids are defined by the functional groups attached to this basic structure. Substituents and hydrogens are designated according to the number of the carbon atom to which they are attached. These are labelled α or β according to their positioning above or below the plane. Cortisol is defined by its hydroxyl group at position 11 while in comparison corticosterone differs only with the absence of a hydroxyl group at position 17.

Figure 1-1: Basic steroid structure

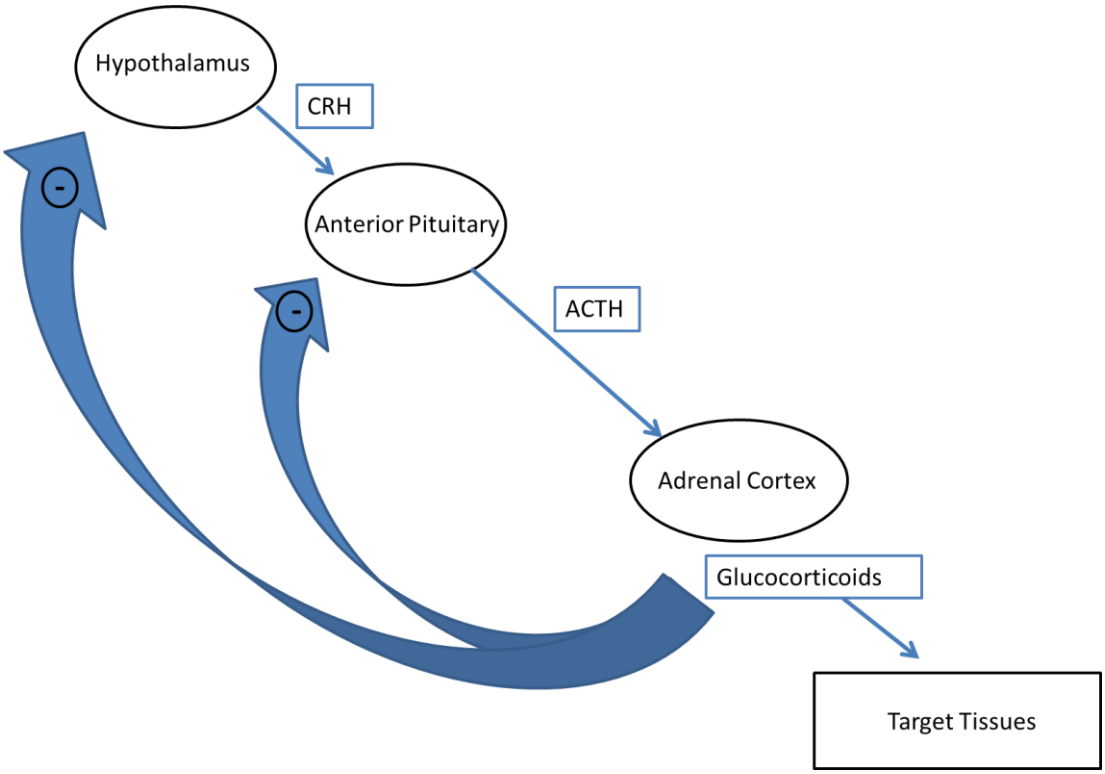


1.1.2 The hypothalamic-pituitary-adrenal (HPA) axis

Glucocorticoids are produced in the adrenal cortex by the zona fasciculata and reticularis. They are under tight regulation by the hypothalamic-pituitary-adrenal (HPA) axis which is a negative feedback system (Figure 1-2). Highly lipophilic, they cannot be stored in vesicles and must be produced *de novo* when required. Corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) are secreted from the paraventricular nucleus (PVN) of the hypothalamus which stimulates release of adrenocorticotrophic hormone (ACTH) from the pituitary gland via activation of pituitary pro-opiomelanocortin (POMC) gene transcription (Vegiopoulos & Herzig 2007). ACTH acts at the adrenal cortex to stimulate steroidogenesis. Glucocorticoids regulate their own production through a number of negative feedback mechanisms. There is inhibition of the synthesis and release of ACTH from the anterior pituitary via interference with POMC transcription and in addition, inhibition of CRH gene expression and secretion in the hypothalamus. This is through direct modulation of neuronal activity in the PVN and other areas of the brain such as the hippocampus, amygdala and prefrontal cortex.

In the zona fasciculata, ACTH binds to the melanocortin type-2 receptor (MC2R), leading to activation of adenylyl cyclase and subsequent increase in cyclic adenosine mono phosphate (cAMP) which activates downstream pathways such as protein kinase A (PKA) (Spiga & Lightman 2015). Activation of the PKA pathway causes rapid synthesis of glucocorticoids within the cell via a number of enzymatic reactions (Figure 1-3).

Figure 1-2: Hypothalamic-pituitary-adrenal axis

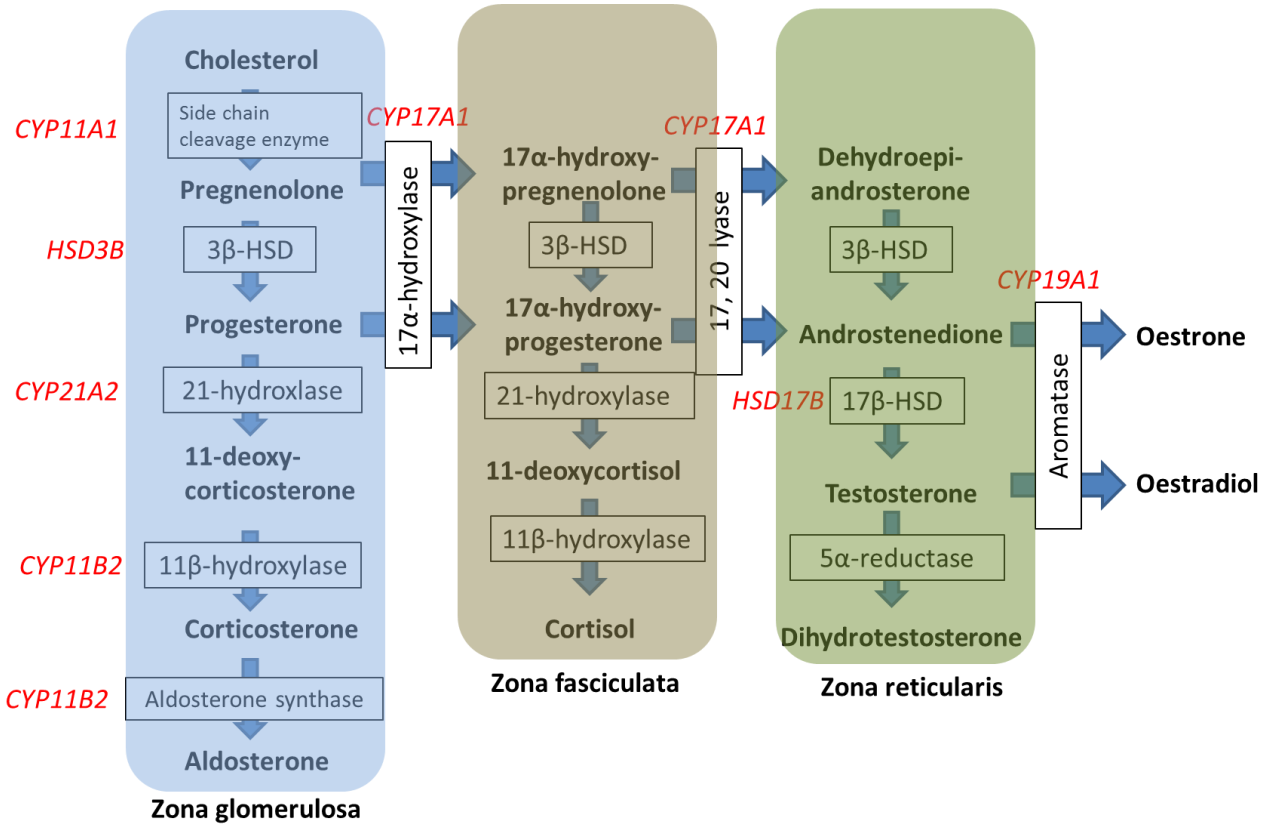


This process is regulated by steroidogenic acute regulatory protein (StAR) which controls delivery of cholesterol to the mitochondria where steroidogenesis occurs (Clark et al. 1994). This is a rate-limiting step, without which, steroidogenesis is severely impaired which manifests clinically as congenital lipoid adrenal hyperplasia (Bose et al. 1996). Cholesterol is converted to pregnenolone by side-chain cleavage enzyme, the product of *CYP11A1*. Pregnenolone is then converted to progesterone by 3β-hydroxysteroid dehydrogenase encoded by *HSD3B1*. 11-deoxycorticosterone is produced from progesterone through enzymatic conversion by 21-hydroxylase from *CYP21A2*.

Thereafter, corticosterone and aldosterone are produced after enzymatic conversion by 11β-hydroxylase and aldosterone synthase respectively, both produced by *CYP11B2*.

This occurs primarily in the zona glomerulosa whereas in the zona fasciculata, 17α-hydroxylation of pregnenolone and progesterone leads to a parallel pathway. 17-hydroxypregnenolone is converted to 17-hydroxyprogesterone by 3β-hydroxysteroid dehydrogenase and further to 11-deoxycortisol by 21-hydroxylase. Cortisol is produced from the final conversion of 11-deoxycortisol by 11β-hydroxylase (Hum & Miller 1993).

Figure 1-3: Steroidogenesis pathway



1.1.2.1 Pattern of glucocorticoid release

Glucocorticoids are released in a pulsatile manner with both circadian and ultradian rhythm. This roughly 24 hour cycle exists in many organisms and aims to achieve homeostasis by anticipation and preparation for changes in environment (Panda et al.

2002). The suprachiasmatic nucleus (SCN) is responsible for driving this by receiving light signals via the retino-hypothalamic tract, triggering a signalling cascade within SCN neurons. This alters gene expression (Dickmeis 2009) and augments the CRH drive through afferents projecting to the paraventricular nucleus and median eminence (Engeland & Arnhold 2005).

Glucocorticoid release varies according to the timing of activity and therefore the circadian peak occurs in the early morning in humans and in the early night in rodents (Dickmeis 2009; Lightman & Conway-Campbell 2010). In humans, the pattern of glucocorticoid release begins to rise around 3am, reaching a peak at approximately 9am before gradually falling throughout the day to a nadir around midnight (Henley & Lightman 2014).

Within the circadian rhythm, there is a further pulsatile profile, the ultradian rhythm, characterised by distinct pulses at approximately 1-hour intervals. There is considerable inter-individual variability in pulse frequency and amplitude and these are subject to further change by normal physiological events such as puberty, lactation and ageing (Windle et al. 1997; Evuarherhe et al. 2009; Conway-Campbell et al. 2012). The origin of this pulsatility was assumed to be hypothalamic however pulsatility was observed to be retained in sheep after hypothalamic disconnection, suggesting a peripheral source (Engler et al. 1990). A mathematical model has since been proposed describing the linear relationship between the delay in feed forward signal from the pituitary to the adrenal and the rapid non-linear feedback from the adrenal to the pituitary (Walker et al. 2010). This suggests an innate oscillatory system which exists solely between the pituitary and adrenal and is crucially dependent on the rate of

production and clearance of glucocorticoid. The significance of pulsatile ACTH release was established by Spiga et al where pulsatile but not continuous infusion of ACTH at equivalent doses to HPA-suppressed rats resulted in pulsatile corticosterone release (Spiga et al. 2011).

Disturbances in ultradian dynamics of the HPA axis have been observed in a number of medical conditions (Henley & Lightman 2014). Frequent blood sampling in patients with obstructive sleep apnoea demonstrated ACTH and cortisol pulses of prolonged amplitude resulting in elevated total cortisol production. Interestingly, these changes were ameliorated after treatment with positive airways pressure after 3 months (Henley et al. 2009). In Cushing's syndrome, there was greater irregularity of release of both cortisol and ACTH when compared to healthy controls (van den Berg et al. 1997). Depression is associated with enhanced pulse magnitude which results in loss of circadian rhythm and changes the pattern of exposure of glucocorticoids to tissues (Young et al. 2004).

It remains unclear whether these changes in HPA pulsatile patterns are caused by or as a consequence of these chronic conditions. It does seem clear that changes in glucocorticoid exposure through changes in secretory profile has an effect on metabolic, cognitive and behavioural well-being. It has been hypothesised that alterations in ultradian rhythms may contribute to the pathogenesis of cardio-metabolic risk (Sarabdjitsingh et al. 2012; Henley & Lightman 2014).

1.1.3 Glucocorticoid transport

Transport of glucocorticoids to target receptors is principally via corticosteroid binding globulin (CBG), a monomeric glycoprotein synthesised mainly in the liver (Gagliardi et al. 2010). Under normal conditions, 80-90% of cortisol is bound to CBG, with 10-15% bound to albumin with lower affinity and the remaining 5-10% unbound or free. Only free cortisol is biologically active. At peaks of cortisol release, CBG is saturated and therefore the free portion increases exponentially above 400-500nmol/l (Ballard 1979). CBG acts as a buffer during secretory surges of glucocorticoid release and as a reservoir during times of trough (Hammond et al. 1990). In times of acute inflammation, CBG is cleaved locally by neutrophil elastase to release cortisol and allow anti-inflammatory effects (Lin et al. 2010).

1.1.4 Receptor binding and signalling

Glucocorticoid biological activity is mediated by two nuclear receptors. The mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) are members of the nuclear receptor subfamily 3, group C, located on chromosome 5. They are closely related to the progesterone and androgen receptors, sharing a common domain structure (Weikum et al. 2017). Both MR and GR remain in the cytoplasm until activated whereupon they translocate to the nucleus and bind to DNA sequences (MR and GR response elements) or other DNA-bound transcription factors to allow trans-activation or trans-repression of target genes (Moraitis et al. 2016). In addition, non-genomic effects are mediated in the cytoplasm and mitochondria (Groeneweg et al. 2011) . It is increasingly recognised that GR is highly dynamic in action and this

stochastic mode of action links with the pulsatile availability of glucocorticoid *in vivo* (Conway-Campbell et al. 2010).

Despite their commonly used names, both GR and MR recognise a number of natural ligands including cortisol, corticosterone, aldosterone and progesterone. GR is almost ubiquitously expressed whereas MR is present in fewer cell types. Classically MR is present in aldosterone sensitive tissues such as the kidney, colon and parotid (Funder 2005). GR has a relatively low affinity for glucocorticoids (K_d =10-25 nM) suggesting it plays a key role when glucocorticoid levels peak such as in the morning or during times of stress (Reul & de Kloet 1985; Hellal-Levy et al. 1999). In contrast, MR has a higher affinity for both glucocorticoids and aldosterone (K_d = 0.5-2 nM) and therefore acts at times of lower glucocorticoid levels in the evening and overnight (Arriza et al. 1987; Hellal-Levy et al. 1999).

The more abundant glucocorticoid in humans, cortisol, circulates roughly 1000-fold higher concentrations than aldosterone. The presence of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) plays a critical role in protecting MR from over-activation by glucocorticoids in target tissues such as the kidney (Funder et al. 1988; Edwards et al. 1987). This enzyme catalyses the nicotinamide adenine dinucleotide (NAD^+)-dependent oxidation of cortisol to its inactive form, cortisone thereby allowing aldosterone to bind to MR in the presence of much higher concentrations of glucocorticoid (Stewart et al. 1988).

Tissue glucocorticoid levels are also controlled by another isozyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) which acts predominantly as a reductase *in vivo*, converting inactive cortisone to cortisol. This allows tissue-specific

regeneration of glucocorticoids independently of HPA axis production, particularly in tissues such as liver, skeletal muscle and adipose (Seckl & Walker 2001). Dysregulation of these enzymes has significant impact on cortisol clearance in a variety of clinical settings. Inactivating mutations in 11β-HSD2 result in the disorder of apparent mineralocorticoid excess where MR are activated by glucocorticoids, which are not inactivated by 11β-HSD2, in MR target tissues manifesting in sodium and water retention, hypokalaemia and severe hypertension (Stewart et al. 1988; Stewart et al. 1987). Conversely, inactivation of 11β-HSD1 or 'relative cortisone reductase deficiency' leads to a relative excess of cortisone compared to cortisol and results in impaired negative feedback of the HPA axis. This manifests clinically with hirsutism as a result of adrenal androgen excess (Phillipov et al. 1996; Jamieson et al. 1999).

1.1.5 Negative feedback of the HPA axis

Negative feedback of the HPA axis is a vital function of the system, aiming to limit exposure to the catabolic actions of glucocorticoids. There are distinct time domains in which this occurs. Fast feedback is measured in seconds to minutes while intermediate and slow effects occur over hours to days. The rapidity of this initial response suggests protein synthesis is not involved and therefore appears to be mediated by non-genomic signalling pathways (Evanson et al. 2010). In contrast, delayed negative feedback is mediated by changes in gene expression through activation of GR and MR (Keller-Wood & Dallman 1984).

Glucocorticoid feedback can occur at the level of the pituitary, hypothalamus and within the hippocampus, paraventricular thalamus and pre-frontal cortex (Hill &

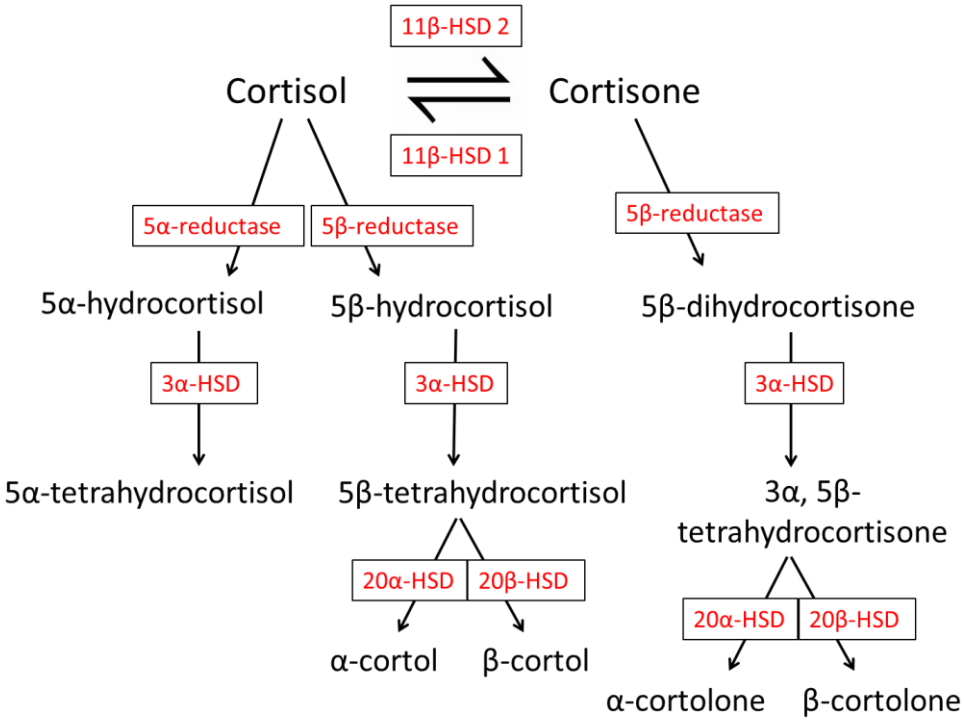
Tasker 2012; Spiga & Lightman 2015). At the pituitary, glucocorticoids act initially to suppress the release of ACTH via rapid feedback effects and thereafter to inhibit synthesis of ACTH via genomic mechanisms (Jones et al. 1977). Within the brain, there is a complex process of rapid and delayed effects acting on the PVN to suppress secretion of CRH and down-regulation of CRH and vasopressin mRNA transcription respectively (Hill & Tasker 2012). The involvement of the higher limbic areas in negative feedback appear to be limited to psychological rather than physical stimuli (Furay et al. 2008). In contrast, brainstem structures influence the HPA axis through detection of physiological stressors such as inflammation or hypotension (Ulrich-Lai & Herman 2009).

1.1.6 Clearance

Glucocorticoids are cleared irreversibly from the circulation via the A-ring reductase enzymes, 5α- and 5β-reductase, acting primarily in the liver to form the dihydro-product (Nixon et al. 2012). These metabolites are then reduced further by 3α-hydroxysteroid dehydrogenase (3α-HSD) and excreted mainly as the tetrahydro-corticosteroids while a proportion are further reduced by 20α/β-hydroxysteroid dehydrogenase (20α/β-HSD) to form cortols and cortolones (Figure 1-4). These metabolites are conjugated with glucuronic acid or sulphate to facilitate renal excretion.

Analysis of 24-hour urinary excretion in healthy individuals suggests 50% of secreted cortisol is excreted as α/β-tetrahydrocortisol and cortisone, 25% as cortols and cortolones, 10% as C19 steroids and 10% as cortolic and cortolonic acids. The remaining portion is excreted as free unconjugated steroids (Tomlinson et al. 2004).

Figure 1-4: Metabolism of cortisol



1.2 Measuring glucocorticoids in humans

1.2.1 Plasma

Measurement of glucocorticoids in plasma or serum is the most common method of assessing adrenal function in humans. Given the circadian rhythm of glucocorticoids, timing of sampling is significant and given circulating concentrations are influenced by production and clearance, these parameters must be considered when interpreting random samples. Early morning samples can be used clinically to determine adequate glucocorticoid levels while late night samples are useful in excluding glucocorticoid excess. It can be difficult to draw any conclusion regarding glucocorticoid metabolism from a single plasma measurement however and the gold standard for assessing HPA axis function is through dynamic testing. Adrenal insufficiency can be assessed by an insulin tolerance test however recent clinical guidelines favour the short synacthen test as a more reliable and practical alternative (Wood et al. 1965; Bornstein et al. 2016). Synthetic $ACTH_{1\text{-}24}$ is given by intramuscular or intravenous injection and a plasma or serum cortisol measurement is taken at baseline and 30 minutes after the injection. The threshold for an adequate response is determined locally.

Cortisol measured in peripheral blood provides a value for the total circulating pool which includes both bound and unbound fractions. This may become clinically relevant in patients with conditions affecting plasma protein concentrations such as liver cirrhosis. The fraction of bound cortisol is less and therefore lower total concentration is measured. As a result of higher circulating free cortisol, there is increased negative feedback and a resultant fall in HPA drive. In these patients, free plasma concentration is more indicative of adrenal function (Fede et al. 2012). The

free portion of plasma cortisol can be measured by ultrafiltration, equilibrium dialysis or gel filtration but these procedures are time consuming and complex and are therefore unsuitable for routine use. An alternative method has been performed by quantifying total plasma cortisol and plasma CBG and calculating free cortisol, but is limited due to the variable binding affinity for CBG (Turpeinen & Hämäläinen 2013).

The analytical method of detecting cortisol has largely involved immunoassay, gas chromatography mass spectrometry (GC-MS) or liquid chromatography tandem mass spectrometry (LC-MS/MS). Immunoassays are particularly susceptible to cross reactivity with both endogenous and exogenous steroids of similar structure leading to falsely elevated results (Shackleton 2010). LC-MS/MS has the advantages of high sensitivity, specificity and high throughput (Hawley & Keevil 2016). Unlike immunoassay, LC-MS/MS allows simultaneous measurement of whole steroid profiles in a single run. Despite this, few clinical laboratories employ LC-MS/MS for cortisol analysis due to the long run times, extensive sample preparation and expense (Taylor et al. 2015). In contrast, glucocorticoid assessment by LC-MS/MS is increasingly becoming a necessity in clinical research as the superior sensitivity and selectivity allows more accurate assessment of glucocorticoids in various biological samples (Taylor et al. 2015).

1.2.2 Saliva

In recent years, the validity of measuring salivary glucocorticoids has been recognised. Changes in binding proteins such as CBG and albumin can greatly affect total plasma glucocorticoid levels. Saliva is an ultrafiltrate of plasma and reflects the biologically active, non-protein bound pool of glucocorticoids in plasma (Hawley & Keevil 2016).

Salivary cortisol follows a circadian rhythm and late night salivary cortisol is a common screening test for Cushing's syndrome which is highly sensitive and specific (>90%) (Newell-Price et al. 2006). Measuring salivary cortisol has been suggested as an alternative to plasma in dynamic testing of the HPA axis to detect hypoadrenalism (Perogamvros, Owen, et al. 2010) It has also been proposed as a useful monitoring tool for patients taking glucocorticoid replacement therapy such as in Addison's disease or Congenital Adrenal Hyperplasia (Turpeinen & Hämäläinen 2013). As a non-invasive and easy-to-collect biological fluid, salivary cortisol is ideal in paediatric populations. Samples are stable and can be stored at room temperature for 1-2 days (Turpeinen & Hämäläinen 2013).

Salivary cortisol is well characterised in humans and indeed salivary cortisone has been proposed as a superior measure of plasma cortisol concentrations due to the significant activity of 11β-HSD2 in the parotid gland (Perogamvros, Keevil, et al. 2010).

1.2.3 Urine

Urinary cortisol concentration also reflects the unbound portion in the circulation, freely filtered at the glomerulus (Hawley & Keevil 2016). Metabolites of glucocorticoid metabolism may also be measured in urine allowing assessment of clearance. A 24-hour urinary collection of urine is preferred to assess urinary free cortisol to allow for diurnal variation and benefits from the lack of confounding by plasma proteins (Newell-Price et al. 2006). This is recommended as one of the commonly used diagnostic tests in the assessment of Cushing's syndrome (Nieman et al. 2008) Analytical methods include immunoassay, high performance liquid

chromatography (HPLC), GC-MS or LC-MS/MS. As previously described, immunoassay is limited in specificity due to the cross reactivity of the many glucocorticoid metabolites present in urine and often over-estimates true urinary free cortisol values. Solvent extraction and separation by chromatography are necessary to reduce interference and improve accuracy (Turpeinen & Hämäläinen 2013).

1.2.4 Hair

Hair has been used by forensic scientists as a method of detecting drugs and banned substances for many years. While methods to detect both endogenous and exogenous glucocorticoids were developed to detect performance-enhancing doping amongst athletes in the late 1990s and early 2000s, it wasn't until a decade later that this was employed as a technique to assess exposure to endogenous glucocorticoids (Cirimele et al. 2000; Raul et al. 2004; Thomson et al. 2010). Hair measurement has the significant benefit of assessing long term glucocorticoid exposure which is not possible with plasma and saliva assessments.

The mechanism by which glucocorticoids are incorporated into hair is not yet clearly defined. It is likely to involve a combination of diffusion from the blood supply to the hair follicle and absorption from surrounding sebaceous gland secretions and sweat (Meyer & Novak 2012). Hair is sampled in segments of 1cm which corresponds to approximately one month of growth, thereby allowing a timeline of glucocorticoid exposure to be created (LeBeau et al. 2011). There is debate over whether there is a washout effect with samples taken further from the follicle and this appears to depend on the method of sample preparation (Russell et al. 2012). A number of studies have investigated the effects of hair colour, gender and hair treatments (e.g. hair product

use, dyeing etc). Hair colour appears to have no contribution to glucocorticoid concentration however the effect of hair dye use is unclear and may lead to lower glucocorticoid measurement (Manenschijn et al. 2011; Kirschbaum et al. 2009).

Hair cortisol measurements have been shown to correlate with plasma and saliva glucocorticoid concentrations in healthy individuals and patients with known hypercortisolism (Manenschijn et al. 2011; Xie et al. 2011). It is particularly relevant in the assessment of patients with suspected cyclical Cushing's syndrome (Manenschijn et al. 2012). In addition, raised hair cortisol is associated with metabolic syndrome and cardiovascular disease (Stalder et al. 2013; Manenschijn et al. 2013). Measurement of long term glucocorticoid exposure has been of interest in evaluation of chronic stress and has been shown to be a valuable biomarker in assessment of disease (Russell et al. 2012).

1.3 Glucocorticoids in disease

1.3.1 Cushing's syndrome

Cushing's syndrome results from exposure to excess glucocorticoids from an exogenous or endogenous source. There is widespread use of synthetic corticosteroids to treat a myriad of conditions such as rheumatoid arthritis, inflammatory bowel disease and asthma and therefore Cushingoid features due to exogenous use are relatively common. Endogenous sources of glucocorticoid excess are rarer and may be ACTH dependent or independent. The different aetiologies of Cushing's syndrome are described in Table 1-1. Endogenous Cushing's syndrome has an estimated incidence of 0.2-5 per million people per year and a prevalence of 39-79 per million with a female preponderance of 3:1 (Lacroix et al. 2015).

Table 1-1: Causes of endogenous Cushing's syndrome

Type (Proportion)	
ACTH dependent (70-80%)	Cushing's disease Ectopic ACTH Ectopic CRH
ACTH independent (20-30%)	Unilateral adrenal adenoma/carcinoma Bilateral adrenal adenoma/carcinoma Bilateral macronodular adrenal hyperplasia Bilateral micronodular adrenal hyperplasia McCune-Albright syndrome

The clinical features of Cushing's syndrome are summarised in Table 1-2. Many manifestations such as lethargy, weight gain, hypertension and menstrual irregularity are common in the general population which can make diagnosis difficult. Features

such as proximal muscle weakness, skin thinning and bruising and rapid onset of symptoms are more specific (Nieman et al. 2008).

Table 1-2: Clinical features of Cushing's syndrome

Body System	**Clinical Features**
General Appearance	Moon Face Hirsutism with frontal balding Posterior fat pad or 'buffalo hump' Central obesity
Cardiovascular	Hypertension Hypercholesterolaemia
Gastrointestinal	Gastric ulcer
Endocrine	Type 2 diabetes mellitus (T2DM)
Locomotor	Osteoporosis Proximal muscle weakness
Reproductive	Disruption of menstrual cycle Reduced libido
Mood	Anxiety Depression Psychosis
Dermatological	Skin thinning Easy bruising Violaceous striae
Other	Lethargy Increased risk of infection Increased thrombotic risk

Cushing's syndrome, regardless of the aetiology, is associated with increased mortality. Excluding malignant causes, Cushing's syndrome has a standardised mortality ratio (SMR) of between 2-4 (Feelders et al. 2012; Bolland et al. 2011). Cardiovascular deaths are the most common (Lindholm et al. 2001). Considerable morbidity is associated with the common manifestations of obesity, hypertension, diabetes and hyperlipidaemia leading to myocardial infarction, left ventricular dysfunction and cerebrovascular disease (Lacroix et al. 2015). The increased risk of

thrombosis also predisposes to thromboembolic disease. In long term remission, cardiovascular risk factors may be improved but do not necessarily return to baseline levels (Geer et al. 2012; Feelders et al. 2012).

1.3.2 Association of cortisol with metabolic syndrome

Metabolic syndrome is a collective term to describe a constellation of metabolic risk factors such as hypertension, hypercholesterolaemia, obesity and type two diabetes mellitus (T2DM) (Reaven 1988). The metabolic changes found in these conditions mimic those seen in Cushing's syndrome and Bjorntorp et al hypothesised that subtle changes in the HPA axis may link pathogenesis and adverse metabolic outcomes in these conditions (Bjorntorp et al. 1999).

Data from a number of studies support the association of hyperactivity of the HPA axis and the development of metabolic syndrome. These associations are demonstrated with significant increases in fasting cortisol levels, urinary free cortisol excretion, response to dexamethasone suppression test and lack of cortisol pulsatility (Phillips et al. 1998; Weigensberg et al. 2008; Pasquali et al. 2002; Rosmond et al. 1998).

1.3.2.1 Obesity

While metabolic syndrome as a spectrum of disease is associated with raised morning plasma cortisol levels, obesity has been associated with normal or even lower morning cortisol levels despite increased cortisol production (Reynolds et al. 2001; Abraham et al. 2013). This paradoxical finding may be explained by a change in metabolic clearance rate of cortisol (Strain et al. 1982) with evidence of increased clearance in rodent models (Livingstone et al. 2000). Human studies of cortisol metabolism in

obesity also showed evidence of increased clearance with urinary steroid profiles (Andrew et al. 1998). This appears to be driven by enhanced activity of A-ring reductases in the liver and impaired cortisol regeneration via 11β-HSD1 (Rask et al. 2001).

In most obese rodents, 11β-HSD1 expression is increased in adipose tissue and reduced in the liver (Livingstone et al. 2000). A similar pattern is seen in obese humans (Rask et al. 2001). Clinical studies using micro dialysis have shown increased cortisol generation in subcutaneous adipose tissue (Sandeep et al. 2005). This appears to be balanced by a reduction in hepatic cortisol production however and highlights that there are tissue specific changes in cortisol metabolism (Stewart et al. 1999; Rask et al. 2001; Walker & Andrew 2006).

Enhanced clearance in obesity is also significant when we consider the regulation of ultradian rhythm via the feed-forward interplay between pituitary and adrenal. Subtle changes in glucocorticoid metabolism may have a significant impact on the maintenance of this pulsatile activation of GR. Changes to ultradian rhythmicity has been demonstrated in obstructive sleep apnoea and Cushing's syndrome (Henley et al. 2009; van den Berg et al. 1997). Loss of ultradian rhythmicity of cortisol may be another mechanism by which obesity is associated with adverse outcomes.

1.3.2.2 Insulin resistance

Insulin resistance is a significant metabolic consequence of excess fat deposition and often leads to the development of T2DM. Defined as an inadequate response of target tissues to circulating insulin, this is characterised by reduced glucose uptake by

skeletal muscle, impaired insulin-mediated inhibition of liver gluconeogenesis and reduced inhibition of lipolysis in adipose tissue (Capurso & Capurso 2012).

A number of studies have shown positive association of cortisol with markers of insulin resistance such as fasting glucose and Homeostasis Model Assessment- Insulin Resistance (HOMA-IR) (Walker et al. 2000a; Reynolds et al. 2001; Kamba et al. 2016; Phillips et al. 1998). Interestingly, there is a female predisposition for cortisol and fasting insulin association (Stolk et al. 1996).

1.3.2.3 Hypertension

Hypertension is a common manifestation within the metabolic syndrome and a number of studies have reported an association between cortisol levels and both systolic and diastolic blood pressure (Phillips et al. 1998; Weigensberg et al. 2008; Walker et al. 2000a). The underlying mechanism for this correlation is likely to be multifactorial, secondary to the physiological effects of increased plasma volume, increased peripheral vascular resistance and elevated cardiac output (Anagnostis et al. 2009). A reduction in nitric oxide production limits vasodilation and may also contribute to hypertension (Mitchell & Webb 2002).

1.3.2.4 Dyslipidaemia

Glucocorticoids have complex effects on lipid metabolism resulting in inconsistent associations of dyslipidaemia with Cushing's syndrome (Macfarlane et al. 2008).

There has been no consistent relationship between morning plasma cortisol and HDL cholesterol (Walker et al. 2000b; Maggio et al. 2006; Reynolds et al. 2010). Varma et al found cortisol to positively correlate with high density lipoprotein (HDL)

cholesterol however others have found the opposite effect (Varma et al. 1995; Fraser et al. 1999). Triglycerides have positive associations with cortisol in most studies (Phillips et al. 1998; Ward et al. 2003) with a few exceptions (Maggio et al. 2006; Abraham et al. 2013).

1.3.2.5 Metabolic syndrome

Changes in 11β-HSD1 have been observed in both animal and human studies of metabolic syndrome. Mouse models over-expressing 11β-HSD1 selectively in adipose tissue have normal systemic serum corticosterone but increased adipose corticosterone concentrations and develop a metabolic syndrome phenotype (Masuzaki et al. 2001). This finding led to the hypothesis that in humans, increased local cortisol concentrations in adipose tissue drive the development of metabolic syndrome and are therefore a potential target for manipulation. 11β-HSD1 inhibitors have been developed by our group (Hughes et al. 2008) and have entered phase 2 trials. A number of pharmaceutical agents have been studied with limited effect on glycated haemoglobin (HbA1c), blood pressure and body weight but no effect on fasting blood glucose (Rosenstock et al. 2010; Shah et al. 2011; Neghab et al. 2015). These relatively limited and disappointing outcomes might be secondary to the bidirectional activity of 11β-HSD1 (Hughes et al. 2012; Anderson 2017).

1.3.3 Congenital adrenal hyperplasia

Congenital adrenal hyperplasia (CAH) is the most common genetic endocrine condition characterised by disruption of the steroidogenesis pathway. The incidence of classic CAH in Caucasian populations ranges from 1:10 000 to 1:20 000 births while non-classic is more common at 1:2500 live births (Van der Kamp et al. 2001; Speiser

et al. 2010). There are wide variations according to ethnicity however with much lower incidence in Chinese and African American populations and higher in Yupik Eskimos (Lee et al. 2000; Therrell et al. 1998; Pang et al. 1982). The most common mutation accounting for 95% of cases is of the *CYP21A2* gene, causing deficiency of the enzyme 21-hydroxylase (Han et al. 2014). This leads to impaired cortisol synthesis, limited negative feedback suppression of the HPA axis and subsequent build-up of steroidogenesis intermediates leading to excess production of adrenal androgens. Up to 75% of patients also have impaired mineralocorticoid production leading to salt-wasting (Speiser & White 2003). Classification of CAH relates to the severity of the mutation with classic CAH the more severe form and non-classic CAH, the mild or late-onset form. Classic CAH is further classified regarding the presence or absence of aldosterone deficiency as either salt-wasting or simple virilising respectively. Most patients are compound heterozygotes with different mutations on each allele. The phenotype is normally related to the less severe mutation and therefore the residual 21-hydroxylase activity (Merke & Bornstein 2005). There is some correlation between genotype and phenotype with regard to severity of disease, in particular the likelihood of mineralocorticoid deficiency however there is a less convincing correlation with degree of virilisation (Krone et al. 2007).

Diagnosis of classic, salt-wasting 21-hydroxylase deficiency is often made in the first few weeks of life, commonly presenting as either ambiguous genitalia in females or a salt-wasting crisis in either sex. Other manifestations include failure to thrive, precocious pseudo puberty and short stature. Patients with non-classic CAH are

generally diagnosed later in life and often present with hirsutism and menstrual irregularity (Speiser & White 2003).

The aim in treatment of CAH is to replace deficient glucocorticoid and limit the consequences of excess adrenal androgens. Plasma 17 hydroxyprogesterone (17-OHP), testosterone and androstenedione levels are measured as markers of control (Speiser et al. 2010). Management goals change throughout different life periods; growth and development are central in infancy and childhood while limiting cardiovascular risk is key later in life. In addition, patients may wish to optimise fertility during early adulthood. Choice of glucocorticoid and treatment regimen depends on age, gender and desire for fertility (Han et al. 2014).

The key to managing CAH is maintaining a fine balance between adequate glucocorticoid +/- mineralocorticoid replacement and sufficient suppression of adrenal androgen secretion. This is often challenging as strictly physiological glucocorticoid replacement doses are often inadequate to suppress excess adrenal androgens and doses which do suppress androgens can result in side effects such as obesity, osteoporosis and cardiovascular disease (Han et al. 2014).

A number of studies have shown both objective and subjective markers of health to be poor in this patient cohort. There is a high prevalence of obesity, hypertension, hypercholesterolaemia and osteoporosis (Arlt et al. 2010). Glucocorticoid treatment regimens varies greatly and there is evidence of overtreatment with suppressed androgen levels, abdominal striae and reduced bone mineral density (Han, Stimson, et al. 2013). Quality of life scores are poor with obesity and compromised sex life a source of anxiety more than short stature or concern about long term health (Han,

Krone, et al. 2013). In male patients, there is increased risk of testicular adrenal rest tumours (TART) which can affect fertility (Han et al. 2014). Mortality in CAH has been reported to be higher than age and sex matched controls with a hazard ratio of 2.3 in males and 3.5 in females. Causes of death included adrenal crisis, cardiovascular disease and cancer (Falhammar et al. 2014).

1.3.4 Addison's disease

Addison's disease is characterised by primary adrenal insufficiency and was first described by Thomas Addison in 1855 (Addison 1855). Addison described primary adrenal failure secondary to tuberculous infiltration however today, autoimmune destruction of the adrenal gland is the most common cause of Addison's disease (Kong & Jeffcoate 1994; Zelissen et al. 1995). It was a fatal condition until corticosteroid preparations became available in the 1930s (Simpson 1938).

Prevalence in white European populations are estimated at 110-140 per million although an even higher prevalence of 220 per million was reported recently in Iceland (M M. Erichsen et al. 2009; Mitchell & Pearce 2012; Olafsson & Sigurjonsdottir 2016). Presentation of Addison's disease is often insidious and non-specific. As evidenced by large case series, clinical features include fatigue, loss of appetite, weight loss and nausea (Erichsen et al. 2009; Mitchell & Pearce 2012). Other more specific features include skin hyperpigmentation, salt craving and postural hypotension. Biochemical abnormalities include hyponatraemia and hyperkalaemia. The gold standard test for diagnosing adrenal insufficiency as advised by the Endocrine Society is the ACTH stimulation or short synacthen test (Bornstein et al. 2016). Management is based on replacement of absent glucocorticoids and mineralocorticoids.

Historically, the prognosis for Addison's disease was considered similar to the background population for those appropriately diagnosed and treated (Mason et al. 1968). More recent studies however have highlighted that standardised mortality rates for patients with adrenal insufficiency are more than double those of age and sex-matched controls (Bensing et al. 2008; Bergthorsdottir et al. 2006). Adrenal crisis remains a significant cause of death, particularly in patients under the age of 40 years (Erichsen et al. 2009). In addition to objective health outcomes, patients with adrenal insufficiency have impaired quality of life with increased anxiety levels and impaired feelings of well-being (Hahner et al. 2007). The cause of higher observed mortality may be due to suboptimal glucocorticoid replacement therapy with supra-physiological doses resulting in increased metabolic and cardiovascular side effects (Bensing et al. 2016).

1.4 Corticosterone in humans

Cortisol is commonly described as the principal glucocorticoid in humans and corticosterone as the principal glucocorticoid in rats and mice. In fact, humans produce both cortisol and corticosterone from the adrenal cortex although cortisol circulates at substantially higher concentration in plasma (Seckl et al. 1990; Karssen et al. 2001; Peitzsch et al. 2015). While corticosterone was included in early human glucocorticoid research, it has increasingly been neglected as cortisol was regarded as the more significant. Corticosterone had been assumed to mimic cortisol in action and effect but to a much lesser extent due to lower circulating concentrations. In fact, both historical and more recent evidence suggests corticosterone has distinct physiology compared to cortisol.

1.4.1 Physiology of corticosterone in humans

Corticosterone was the first steroid isolated from the adrenal gland in 1937 by Hench, Kendall and Reichstein, who were subsequently jointly awarded the Nobel Prize for Physiology or Medicine in 1950 for their work. This has since led to a significant body of research identifying and characterising the actions of each hormone released from the adrenal and in developing sensitive and specific methods to accurately detect these hormones in plasma.

1.4.1.1 Measuring plasma corticosterone

Conn observed in 1950 that corticosterone caused sodium, chloride and water retention and potassium diuresis. He also noted increased resistance to insulin and reduced carbohydrate tolerance. Interestingly, he concluded that corticosterone was a superior substitute replacement therapy compared to cortisone and deoxycorticosterone as

clinical improvement was 'rapid and excellent' and the dose requirement was less (Conn 1950; Conn 1951). A method of chloroform extraction followed by chromatographic quantitation by fluorescence was developed by Sweat and formed the basis of method development for the next 20 years (Sweat 1955). Early studies estimated plasma corticosterone at higher values than we would consider normal today (Sweat 1955; Peterson 1957; Ely et al. 1958) and may reflect the lack of specificity of these early methods. These are summarised in Table 1-3 below.

Radioimmunoassay determination of corticosterone following extraction and separation with chromatography was developed from the 1970s. An increasing demand for rapid throughput assays led to the development of direct immunoassays which sacrificed accuracy and validity for economy of time and money (Handelsman & Wartofsky 2013).

Mass spectrometry became the gold standard for steroid analysis as sensitivity and expense improved throughout the 1980s and 1990s (Shackleton 2010). From the more recent publications, plasma corticosterone circulates at concentrations 10-20 fold less than cortisol at concentrations of approximately 16-40 nmol/L (Seckl et al. 1990; Karssen et al. 2001). Raubenheimer et al reported higher concentrations (~60 nmol/L) however this was in the context of patients about to undergo an invasive procedure (Raubenheimer et al. 2006).

Table 1-3: Literature review of corticosterone measurements in human plasma

Paper	Method of detection	Plasma Corticosterone Values (nmol/L)		Corticosterone: Cortisol	Corticosterone Response to ACTH
		Mean am sample	Diurnal		
Sweat 1955 n=21	Fluorometric Analysis	124.1±66.4	-	0.42	2-5x increase
Peterson 1957 n=30	Fluorometric Analysis with isotope dilution	31.7±11.5	Peak 8am Nadir 12mn Range: 0-43.3	0.08	Increase
Ely 1957 n=20	Fluorometric analysis	86.6±5.8	-	0.28	0.7x increase
Bondy et al 1957 n=29	Paper chromatography and fluorometric analysis	37.5±72.2	-	0.13	Increase
Martin and Martin 1968 n=33	Fluorometric analysis with liquid liquid extraction	17.3±17.3	Peak 8am Nadir 8pm Range: 4-17.3	0.27	6x increase
Fraser and James 1968 n=16	Double isotope derivative assay	20.8±2.3	-	0.06	Increase
Hamanaka et al 1970 n=504	One step elution chromatography	21.1±2.8	Peak 6am Nadir 12mn Range: 0-17.3	0.06	6x increase
West et al 1972 n=15	Solvent extraction Paper chromatography and radioimmunoassay	11.4±6.5	Peak 6am Nadir 1am	0.03	-
Seckl et al 1990 n=7	Radioimmunoassay	~35	Peak 8am Nadir 12mn Range: 10-36	0.1	-
Karssen et al 2001 n=11	LC-MS	16.4±3.14	-	0.06	-
Raubenheimer et al 2007 n=11	Enzyme linked immunosorbent assay	58.4±9.2	-	0.07	-

1.4.1.2 Corticosterone and the HPA axis

While it was noted that cortisol circulated at higher concentration than corticosterone, both remained subject to ongoing investigation and early observations were made of differences in metabolism. Both Peterson and Fraser observed a reduction in the corticosterone: cortisol ratio after ACTH administration and this was further confirmed by Nishida et al in 1977 (Peterson 1957; Fraser & James 1968; Nishida et al. 1977). Some studies found minimal response of corticosterone to ACTH however which perhaps reflects the wide range of analytical methods at the time (Ely et al. 1958). An enhanced response of corticosterone to surgical stress compared to cortisol has also been reported (Fraser & James 1968; Hamanaka et al. 1970) with similar findings after insulin induced hypoglycaemia (Fraser & James 1968). Suppression of corticosterone after dexamethasone administration was blunted compared to cortisol, suggesting that there are differences in the regulation of cortisol and corticosterone secretion (Newsome et al. 1972; Nishida et al. 1977).

1.4.1.3 Pre-receptor metabolism

Similar to cortisol, corticosterone is metabolised by the 11β-HSD enzymes to catalyse the conversion between active and inactive forms. Corticosterone is converted to its inactive derivative, 11-dehydrocorticosterone by NAD^+-dependent 11β-HSD2 (Monder & Lakshmi 1989).

A number of studies have measured the affinity of corticosterone and cortisol for each isozyme of 11β-HSD. There are consistent reports of corticosterone having greater affinity for 11β-HSD2 than cortisol (Albiston et al. 1994; Gong et al. 2008; Maser et

al. 2002). Michaelis-Menten plots were created using this data and suggest that given the basal circulating concentrations of corticosterone and cortisol, 11β-HSD2 inactivation of each glucocorticoid is comparable (Mackenzie 2015).

The activity of 11β-HSD1 depends on the cells or tissue used in analysis. Comparing affinity of cortisone and 11-dehydrocorticosterone in human HEK-293 cells, Arampatzis et al report similar Km of 519±71 and 420±87 nM respectively (Arampatzis et al. 2005). Dissociation constants of 11β-HSD1 in human liver samples for cortisone and 11-dehydrocorticosterone are reported to be 13.9 and 19.7 μM respectively (Maser et al. 2002). Plotting enzyme kinetics, the Km of 11β-HSD1 is above physiological circulating concentrations of both glucocorticoids and at the concentrations studied, cortisol production exceeded that of corticosterone (Mackenzie 2015).

1.4.1.4 Receptor binding

Binding affinity of corticosterone was measured initially in rat studies. De Kloet et al reported corticosterone had the highest affinity for corticosteroid receptors in the rat brain compared to progesterone, deoxycorticosterone (DOC), dexamethasone and aldosterone (de Kloet et al. 1984). Specific binding assays for human MR through molecular cloning in the 1980s compared its affinity for aldosterone, corticosterone, DOC and cortisol and found these were all very similar at approximately 1.3 nM (Arriza et al. 1987). A further study in 1999 found corticosterone had reduced transactivation via MR compared to cortisol but binding affinity was not measured (Hellal-Levy et al. 1999). More recently, Odermatt's group in Switzerland compared

binding affinity of cortisol, corticosterone and aldosterone with wild type and mutant GR and MR. Binding affinities were not statistically different compared between corticosteroids but corticosterone appeared to have higher affinity for MR than cortisol (Ki 0.39 nM vs Ki 1.63 nM respectively) (Mani et al. 2016).

Limited studies have been carried out comparing binding affinity of GR. Giannopolous et al compared binding of different glucocorticoids to GR in a variety of species (Giannopoulos & Keichline 1981). In cortisol-secreting species such as humans and guinea pigs, cortisol had 1.5-3 times higher affinity for GR compared to corticosterone whereas corticosterone-dominant species such as rodents, had a 3-4 fold higher affinity than cortisol. Mani et al found a similar difference in affinity with cortisol binding human GR at Ki 103 nM and corticosterone at Ki 175 nM (Mani et al. 2016).

There are conflicting reports of GR and MR binding affinities in the literature but the most recent data would suggest cortisol has a higher affinity for GR while corticosterone has higher binding affinity to MR.

1.4.1.5 Production and clearance

Cortisol and corticosterone both display a circadian rhythm, peaking in the morning and with a nadir in the evening and overnight (Peterson 1957; Martin & Martin 1968; Hamanaka et al. 1970).

The secretion rate of corticosterone has been estimated using tritium labelled corticosterone and measuring isotope dilution. The estimated rate of production is relatively consistent at approximately 10-11 μmol/day (Peterson 1957; Peterson 1959; New et al. 1969). This is 5-10 fold less than the approximate secretion rate of cortisol,

estimated at between 50-80 μmol/day (Silber et al. 1958; Bondy & Upton 1957; New et al. 1969). The relative difference in production rate is less than the difference in circulating concentration and therefore suggests enhanced clearance of corticosterone. This is consistent with evidence that infusions of corticosterone were reported to have a shorter half-life (0.5-1.5 hours) than cortisol (1.5-2 hours) after bolus infusion (Ely et al. 1958; Peterson 1959; Peterson & Pierce 1960). This is likely to reflect higher affinity for hepatic reductases and less efficient regeneration of corticosterone by 11β-HSD1. A difference in CBG binding may also explain this difference in production rate and circulating concentration however the binding affinity of cortisol and corticosterone for CBG was measured using equilibrium dialysis and found to be comparable (Stroupe et al. 1978).

Clearance of corticosterone follows a similar pathway to cortisol with A-ring reduction in the liver to 5α-tetrahydro products and lesser amounts of 3α,5β-corticosterone excreted in the urine (New et al. 1969). Over and above this however, corticosterone and its 5α-A Ring reduced metabolites are also excreted in the bile, passing into the gut where they are converted to 21-dehydroxylated products 11β-OH-progesterone and 11β-OH-3α,5α-progesterone by anaerobic bacteria (Shackleton et al. 1979).

1.4.1.6 Corticosterone in saliva and hair

Salivary corticosterone is not well studied and there are limited published data to review. McVie et al measured paired plasma and saliva concentrations of cortisol, corticosterone and aldosterone at baseline and after ACTH stimulation. Measured by radioimmunoassay, mean salivary corticosterone was 0.5 nmol/L compared to mean

plasma corticosterone of 12 nmol/L (McVie et al. 1979). Since the concentration of both cortisol and corticosterone are approximately 10-fold less than plasma, immunoassay is limited not only by cross-reactivity with other steroids but also by limit of detection. In a more recent study assessing the effects on exercise on circulating glucocorticoids, salivary corticosterone was not detectable using radioimmunoassay (Del Corral et al. 2016). While we have some indication that salivary corticosterone can be measured from McVie et al, this was with a dated analytical method and provides no information regarding diurnal variation.

A literature search did not reveal any evidence that corticosterone has been measured in hair. The reported values of hair cortisol are low (5-91 pg/mg) (Raul et al. 2004; Manenschijn et al. 2012) and assuming the ratio of cortisol to corticosterone is similar to plasma, it may not be possible to detect with current methods.

1.4.2 Transmembrane transport

As discussed above, corticosterone circulates at lower concentration in plasma, appears to have an enhanced response to ACTH and surgical stress and has a shorter half-life compared to cortisol. In addition to these observations, tissue specific differences in corticosterone and cortisol concentrations have been described, suggesting distinct regulation of transport across the cell membrane.

An interesting observation was made by Karssen et al when investigating the regulation of cortisol at the blood brain barrier. Corticosterone concentrations were relatively higher in post mortem brain tissue samples than in corresponding plasma samples (Karssen et al. 2001). This was supported by findings that corticosterone

concentration in cerebrospinal fluid (CSF) was similarly higher than in plasma (Raubenheimer et al. 2006). Since both cortisol and corticosterone are highly lipophilic and enter cells by diffusion, tissue concentrations should reflect circulating concentrations unless there is active export across the cell membrane. These findings suggest cortisol and corticosterone are under differential transport from cells in a tissue specific manner.

1.4.2.1 ABC transporters

ATP-binding Cassette (ABC) transporters were first identified in the 1980s when investigating multi-drug resistance in cancer patients (Gros et al. 1986). There are 7 sub-groups, named A-G, of which ABCB1 and ABCC1 have significance in glucocorticoid handling (Webster & Carlstedt-Duke 2002).

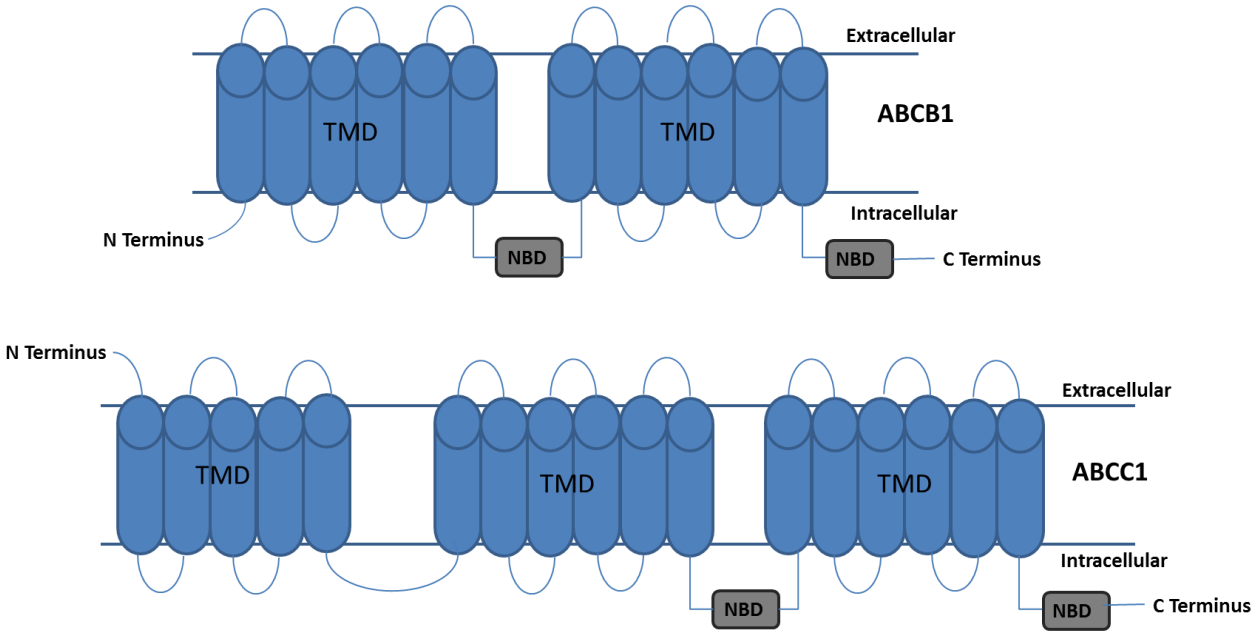
ABC transporters have a typical configuration of two trans-membrane domains (TMD) and two nucleotide binding domains (NBD) as shown in Figure 1-5. The NBD at the C-terminus is involved in hydrolysing adenosine triphosphate (ATP) to facilitate transport of substrates.

ABCB1 is most highly expressed in the adrenal gland but is also expressed in the kidney, brain and small intestine (Nishimura & Naito 2005). Expression of ABCB1 at the blood brain barrier (BBB) is particularly relevant as it plays an important role in protecting the brain from endogenous and exogenous toxins. ABCB1 is principally expressed at the luminal membrane of capillary epithelial cells and acts as a neuroprotective and detoxifying efflux pump (Cordon-Cardo et al. 1989).

Physiologically, ABCB1 exports endogenous substrates as well as ingested or acquired neurotoxic substances which can present a challenge to effective drug delivery (Begley 2004).

Figure 1-5: Molecular structure of ABC Transporters ABCB1 and ABCC1

ABCB1 has two transmembrane domains (TMD) and two nucleotide binding domains (NBD) while ABCC1 has an additional TMD.



Expression of ABCB1 at the BBB limits access of synthetic glucocorticoid, dexamethasone to the brain in rodents (Meijer et al. 1998). Corticosterone, the principal glucocorticoid in rodents, readily gains access to brain in the mouse however cortisol, as the more abundant glucocorticoid in humans, is actively exported at the BBB (Ueda et al. 1992). It has been demonstrated *in vitro* and *in vivo* that ABCB1 limits access of endogenous cortisol but not corticosterone to the brain (Karssen et al.

2001; Webster & Carlstedt-Duke 2002).

ABCC1 has an additional TMD comprising five transmembrane segments (Figure 1-5) and is expressed more widely than ABCB1 (Nishimura & Naito 2005; Yu et al. 2013). Adipose tissue and skeletal muscle have relatively high ABCC1 expression (Nixon et al. 2016) and our unpublished data suggest a similar situation to these tissues with higher ABCC1 expression compared to ABCB1 in human bone and bone marrow (Mark Nixon, unpublished). ABCC1 transports drug conjugates of glutathione, leukotrienes and other organic anions and causes significant resistance to drugs such as doxorubicin and vincristine (Dean et al. 2001). *In vitro* studies have demonstrated that ABCC1 exports corticosterone but not cortisol from a mouse cell line and is inhibited by the ABCC1 inhibitor probenecid (Webster & Carlstedt-Duke 2002). Further, Nixon et al describe ABCC1, but not ABCB1, expression in human adipose tissue and that inhibition of ABCC1 increases intracellular corticosterone in human adipocytes with no effect on intracellular cortisol. Mice with pharmacological and genetic inhibition of ABCC1 accumulated more corticosterone and increased glucocorticoid responsive transcripts in adipose (Nixon et al. 2016).

1.4.2.2 ABCB1 and the HPA axis

The presence of ABCB1 at the BBB plays a significant role in protecting the brain from exposure to toxins. In particular, many drugs are exported at the BBB making some chemotherapy agents less effective. The export of dexamethasone is another example of neuro-protection but the exclusion of endogenous cortisol is more surprising. Since corticosterone is not affected by ABCB1 transport, relative

concentrations within the central nervous system (CNS) are likely to be higher than in the circulation and this was demonstrated in post mortem brain samples (Karssen et al. 2001). This suggests corticosterone may play a more important role in HPA axis negative feedback at the level of the hypothalamus than previously thought. Human *in vivo* experiments confirmed corticosterone was relatively more abundant in CSF than plasma (approximately 40% in CSF and 7% in plasma of total active glucocorticoids) (Raubenheimer et al. 2006). A comparison of ACTH suppression by cortisol or corticosterone in healthy volunteers given metyrapone showed no difference between cortisol and corticosterone at a bolus of 0.15 and 0.04 mg/kg (Raubenheimer et al. 2006). While this study did not support the hypothesis that corticosterone is the predominant centrally-acting glucocorticoid, this may only relate to acute situations, and the study was limited by differences in the pharmacokinetics of cortisol and corticosterone resulting in different plasma exposure of each. With confirmation that there is relatively higher concentration of corticosterone in CSF, there remains the question of whether corticosterone plays a significant role at lower circulating concentrations when MR activation is predominant.

1.4.2.3 ABCC1 in adipose tissue and skeletal muscle

The role of ABCC1 in adipose tissue and skeletal muscle has not been well defined until recently. Nixon et al demonstrated higher expression of ABCC1 than ABCB1 in human adipose tissue of healthy individuals with corticosterone levels below or near the limit of detection (Nixon et al. 2016). The authors went on to test the potency of corticosterone compared to cortisol in inducing metabolic effects via adipose tissue. In a randomised crossover study of patients with Addison's disease, deuterated

glucocorticoids, D4-cortisol and D8-corticosterone, were infused at sequential steady state concentrations for 270 minutes. Again, there was no difference between glucocorticoid for ACTH suppression, nor any difference in metabolic markers including glucose, glycerol, free fatty acids or insulin. However, there was an increase in expression of acutely responsive glucocorticoid transcripts period circadian protein homolog 1 (PER1) and lipoprotein lipase (LPL) in the cortisol group compared to corticosterone (Nixon et al. 2016). While there was no evidence of improved metabolic markers, this was a short-term study and any effect may be limited as a result. This raises the question of whether corticosterone might be a superior choice as a replacement therapy in patients with impaired glucocorticoid synthesis.

1.4.3 Associations of the cortisol: corticosterone ratio with the metabolic syndrome

The metabolic syndrome is strongly associated with increased morbidity and mortality related to cardiovascular disease (Reaven 1988). While lifestyle factors are associated with the development of these conditions, there is also a substantial heritable component. Genome-wide association studies have consistently reported associations between variants in *CYP17A1* gene expression and both obesity and hypertension (Yan et al. 2012; Newton-Cheh et al. 2009). *CYP17A1* encodes the enzyme 17-hydroxylase in the steroidogenesis pathway and is therefore integral in the synthesis of cortisol and any variation in expression will affect the ratio of cortisol to corticosterone (Figure 1-2).

The mechanism underlying this association is not well defined. Deficiency of 17-hydroxlyase results in low renin hypertension due to the accumulation of mineralocorticoids deoxycorticosterone and aldosterone (Goldsmith et al. 1967). It could be assumed therefore that such polymorphisms result in reduced production of this enzyme.

While hypertension is a key component of the metabolic syndrome, genetic risk factors for increased blood pressure are not associated with all other features of this syndrome. In fact, variations in *CYP17A1* are associated with a reduction in both subcutaneous and visceral fat and these polymorphisms which predict obesity are associated with insulin resistance (Hotta et al. 2012; Echiburú et al. 2008). This presents a paradox of *CYP17A1* polymorphisms causing a reduction in 17-hydroxylase and increased corticosterone to cortisol ratio being associated with an increase in blood pressure but lower BMI and improved insulin sensitivity.

This may be explained by the evidence presented above regarding differential control of the HPA axis centrally. Cortisol is exported from the CNS by ABCB1 whereas corticosterone is not. Therefore it appears to be changes in corticosterone concentration which influence the drive of the HPA axis and effect the circulating cortisol concentration. A relative excess of corticosterone would reduce HPA activation and consequently cortisol production whereas relative deficiency would drive HPA axis activation and cause cortisol excess. The metabolic syndrome is associated with increased activation of the HPA axis and resultant cortisol excess.

There is good evidence that raised early morning cortisol is associated with many features of the metabolic syndrome, such as hypertension, dyslipidaemia and insulin resistance (Phillips et al. 1998; Reynolds et al. 2003; Reynolds et al. 2010). There is limited evidence however of the associations of raised morning corticosterone although we would hypothesise that raised corticosterone would be associated with a favourable metabolic profile compared to cortisol.

There are no published data regarding the association of corticosterone and metabolic parameters however this was addressed in a recent thesis (Mackenzie 2015). A number of UK based population cohorts were investigated: the Orkney Complex Disease Study (ORCADES); the Edinburgh Type 2 Diabetes Study (ET2DS) and the East Hertfordshire Study (EHERTS).

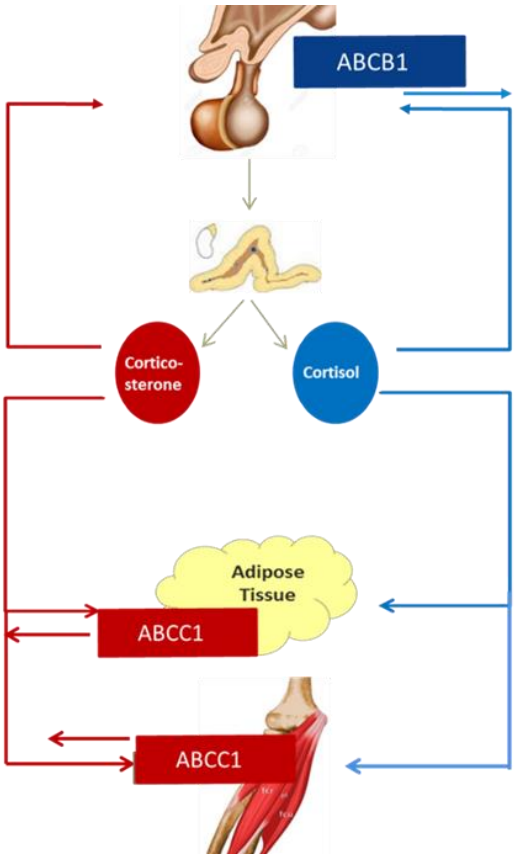
In the ORCADES study, a genetic epidemiological study based in the Orkney islands, both raised morning plasma cortisol and corticosterone were associated with lower body mass index (BMI), raised fasting glucose and raised triglycerides although the magnitude of effect was greater with cortisol. Neither cortisol nor corticosterone was associated with total cholesterol or low density lipoprotein (LDL) cholesterol however raised morning corticosterone was associated with HDL cholesterol. In patients with established T2DM in ET2DS, raised plasma cortisol and corticosterone was associated with lower BMI and an increase in fasting glucose. After dexamethasone suppression in EHERTS, higher corticosterone was associated with lower BMI but also a lower fasting insulin and HOMA-IR. After ACTH stimulation, higher corticosterone was associated with lower glucose before and after glucose load and a lower HOMA-IR.

Associations from the ORCADES and ET2DS confirmed previous findings of cortisol associations with features of metabolic disease and corticosterone largely mimics these associations. Data from EHERTS is intriguing due to the effective normalisation of the HPA axis with dexamethasone induced suppression followed by ACTH activation. There was an inverse association with glucose and HOMA-IR. If corticosterone does make a disproportionate contribution to HPA axis suppression, these results provide a key observation into the regulation of the HPA axis in humans.

1.4.4 Alternative HPA axis

Drawing together the evidence presented above, we propose an alternative HPA axis (Figure 1-6). We hypothesise that while cortisol circulates in higher concentration than corticosterone in plasma, differential tissue specific regulation of ABCB1 and ABCC1 allows corticosterone to exert a more significant role in HPA negative feedback. The greater expression of ABCB1 in brain excluding cortisol but not corticosterone is supported by relatively higher concentrations of corticosterone in brain tissue and CSF (Karssen et al. 2001; Raubenheimer et al. 2006). With ABCC1 highly expressed in skeletal muscle and adipose tissue, cortisol appears to be the principal glucocorticoid acting in these tissues (Nixon et al. 2016).

Figure 1-6: Alternative negative feedback of the HPA axis



With this hypothesis in mind, the association of variants in *CYP17A1* with hypertension and obesity may be explained by an increasein 17-hydroxylase activity and therefore relative corticosterone deficiency. This would drive up-regulation in negative feedback of HPA axis and as a result, relative cortisol excess. This would account for higher morning plasma cortisol values in metabolic syndrome, an association not reflected in morning corticosterone concentrations.

Implications of this alternative axis are particularly relevant in glucocorticoid replacement therapy. Current cortisol-based and synthetic glucocorticoids require relatively high doses to suppress ACTH and accumulation within adipose tissue may

result in over-activation of corticosteroid receptors. This, in turn, may lead to the development of glucocorticoid induced features of the metabolic syndrome. If corticosterone were the basis for glucocorticoid replacement therapy, relatively lower doses would be required for ACTH suppression and accumulation in adipose tissue and skeletal muscle would be less due to the presence of ABCC1.

1.5 Glucocorticoid replacement therapy

1.5.1 Current glucocorticoid replacement therapy

Current glucocorticoid replacement therapies are based on cortisol and the most commonly used replacement is hydrocortisone (Forss et al. 2012). Hydrocortisone is rapidly absorbed in the gut when taken orally and has a bioavailability of over 90%. Time to maximum concentration is only 1-2 hours and the half-life is short at 1.8-2 hours (Thomson et al. 2007; Buning et al. 2017).

Prednisolone has an intermediate duration of action and has more anti-inflammatory action than hydrocortisone (Meikle & Tyler 1977). Dexamethasone is a selective GR agonist and therefore has no mineralocorticoid activity. Prednisolone has a longer half-life than hydrocortisone of 2-4 hours while dexamethasone is longer acting at 4-5 hours (Johannsson et al. 2007; Williams et al. 2016). Cortisone acetate is used more frequently in Europe and North America and has a slightly delayed onset of action compared to hydrocortisone as it needs to be activated by hepatic 11β-HSD1 (Oelkers et al. 2001; Laureti et al. 2003). The biological half-life of cortisone acetate is longer than hydrocortisone however (Feek et al. 1981).

There are no randomised controlled drug trials in Addison's disease or CAH and current guidelines for optimal treatment are based on low quality evidence. In Addison's disease, hydrocortisone at doses of 15-25 mg or cortisone acetate 20-35 mg in two to three daily doses are suggested with prednisolone 3-5 mg daily as an alternative (Bornstein et al. 2016). It is suggested to avoid the use of dexamethasone due to difficulties in dose titration leading to increased risk of Cushingoid side effects.

In adults with CAH, Endocrine Society guidelines suggest treatment with hydrocortisone or long-acting glucocorticoids (Speiser et al. 2010). In a UK cohort study of 203 patients with CAH, glucocorticoid therapies were varied: hydrocortisone (26%), prednisolone (43%), dexamethasone (19%) or a combination (10%). Timing of dosing was also varied with reverse circadian administration in 41% (Arlt et al. 2010). In an association study of health outcomes, increasing glucocorticoid dose was associated with increased blood pressure although without significant improvement in markers of disease control. Any combination of treatment including dexamethasone resulted in improved disease control but at the expense of increased association with insulin resistance (Han, Stimson, et al. 2013). This study suggests that increasing the dose of glucocorticoid does not necessarily result in better disease control and may result in more side effects. It also highlighted the wide variety of treatments in use, underlining the lack of consensus on optimal glucocorticoid treatment.

In a survey of patient-perceived health outcomes in 1245 patients with adrenal insufficiency, a majority reported significant impact of their disease or treatment on subjective health. Most patients took hydrocortisone (75%) either twice (42%) or thrice (32%) daily. 76% of respondents were concerned about long term side effects including osteoporosis, obesity and cardiovascular disease (Forss et al. 2012).

1.5.1.1 Modified and sustained release glucocorticoids

The increased morbidity and mortality in both Addison's disease and CAH has been hypothesised to be secondary to a combination of lack of natural glucocorticoid circadian rhythm and supra-physiological dosing. In an attempt to mirror natural

circadian release of glucocorticoids, modified release and delayed release preparations of hydrocortisone have been developed and tested in patients with adrenal insufficiency.

Various attempts have been made to more accurately reflect physiological glucocorticoid release with three times daily dosing and a continuous subcutaneous infusion which resulted in improved subjective quality of life and reduced overall daily dose (Groves et al. 1988; Løvås & Husebye 2007). Pilot studies were also carried out comparing conventional replacement regimens with two to three times daily hydrocortisone and varied intravenous infusion rates. It was demonstrated that variable infusion of hydrocortisone resulted in better control of ACTH and 17-OHP in patients with Addison's and CAH (Merza et al. 2006). This lead to the development of two modified release preparations of hydrocortisone: Chronocort® (Diurnal Ltd, Cardiff, UK) and Plenadren® (Shire International, Jersey, UK).

Chronocort®, a modified and delayed release preparation of hydrocortisone, was tested in healthy individuals to determine pharmacokinetic profile. The initial study in 6 healthy individuals identified a formulation of hydrocortisone with delayed drug release of 4 hours and median peak cortisol concentration of 10 hours (Newell-Price et al. 2008). This formulation was then tested in healthy volunteers to compare cortisol profiles with conventional immediate release hydrocortisone. This suggested modified release hydrocortisone 15-20mg at 11pm and 10mg at 7am could reproduce physiological cortisol levels (Debono et al. 2009). In comparison with conventional hydrocortisone therapy in patients with CAH, better control of early morning

elevations in ACTH and 17-OHP were demonstrated however daytime cover was inadequate on once daily dosing (Verma et al. 2010). A further phase 2 study with 6 months of treatment continued to show more effective control of ACTH and adrenal androgens but there was a significant fall in bone mineral density and rise in osteocalcin and HOMA-IR (Mallappa et al. 2015).

Plenadren®, a dual release, once daily hydrocortisone tablet, comprising an immediate-release coating and extended release core was developed to attempt to reproduce the physiological profile of cortisol release. Studies in healthy individuals established the plasma pharmacokinetic profile was similar to the physiological release of cortisol (Johannsson et al. 2009). In comparison with three times daily immediate release hydrocortisone, modified release hydrocortisone was associated with a 20% lower bioavailability with higher concentration in the morning and lower in the evening. There was a significant reduction in HbA1c, weight and both systolic and diastolic blood pressure (Johannsson et al. 2012). This study was limited by its short follow up time of 12 weeks and lack of effective blinding to interventions. Comparisons with immediate release hydrocortisone were invalid due to the differences in bioavailability. The average daily dose of hydrocortisone of at least 30mg was higher than recommended by Endocrine Society guidelines (Bornstein et al. 2016).

While extended release preparations have been associated with higher quality of life scores, most studies are of too short follow up to allow assessment of cardiovascular outcomes or effect on bone mineral density. As a result, none of these preparations have been adopted into routine clinical practice until evidence of their improved

effectiveness compared to current standard therapy is more robust. A randomised controlled trial comparing Plenadren® with standard hydrocortisone over a longer period is due to be reported soon (Clinical Trials identifier NCT 02277587).

1.5.1.2 Continuous subcutaneous hydrocortisone infusion (CSHI)

There has been further development in delivering glucocorticoid replacement therapy as a continuous subcutaneous infusion. A prospective randomised crossover study in Norway and Sweden compared three times daily hydrocortisone with a 24-hour variable subcutaneous infusion over a 12 week period (Øksnes et al. 2014). Morning ACTH and salivary cortisol profiles were improved in the infusion phase however an overall higher dose of hydrocortisone was infused and this was associated with higher morning glucose with a trend for higher BMI and HOMA-IR. A similar study comparing CSHI with standard oral hydrocortisone in Addison's disease was carried out in Australia although blinded and over a shorter follow up of 4 weeks (Gagliardi et al. 2014). Doses of infused and oral hydrocortisone were effectively matched but the primary outcome of subjective health status was no different between groups.

A more recent study has studied the use of CSHI in a small number of CAH patients in an open label design over a 6 month period (Nella et al. 2016). Markers of CAH control were improved compared with baseline but remained above target levels. Metabolic outcomes were relatively unchanged and positive effects on quality of life and fatigue were reported.

Further studies are required to establish whether CSHI is an effective alternative form of glucocorticoid replacement therapy with larger cohorts of patients over a longer follow up.

1.5.1.3 Adjuncts to glucocorticoid treatment

Alternative approaches to treatment have been suggested for CAH with different levels of the steroidogenesis pathway targeted. Among glucocorticoid dose sparing strategies, CRH antagonists and ACTH inhibitors have been proposed with some early clinical trials conducted (Holsboer & Ising 2008; Schteingart 2009).

Limiting androgen secretion is traditionally done through negative feedback loops but other strategies include targeting synthesis, conversion of testosterone, aromatisation and receptor activity. Ketoconazole is the most widely studied adrenal androgen inhibitor but is limited in use by side effects and poor oral absorption (Hsieh & Ryan 2008).

Abiraterone acetate is a prodrug which is metabolised to abiraterone, a potent inhibitor of *CYP17A1*. In prostate cancer, this treatment is given to induce chemical castration by inhibiting testosterone production and has been shown to improve survival in castration-resistant cases (de Bono et al. 2011; Ryan et al. 2013). Pharmacological inhibition results in the same phenotype as in 17 hydroxylase deficiency with low renin hypertension due to high circulating DOC and aldosterone. In order to manage this, abiraterone treatment is combined with prednisolone to suppress ACTH driven HPA axis overactivity. The mineralocorticoid excess experienced in these patients due to excess production of DOC is absent in CAH and therefore abiraterone added to

physiological glucocorticoid and mineralocorticoid replacement is hypothesised to control androgen excess. Early phase 1 clinical studies in patient with CAH have been promising and further studies are in progress (Auchus et al. 2014).

1.5.2 Corticosterone as an alternative glucocorticoid replacement therapy

To date, innovations in glucocorticoid replacement therapy have focussed on improving pharmacokinetic profile. All currently available glucocorticoids contain either cortisol (hydrocortisone), cortisone (converted to cortisol) or a synthetic glucocorticoid and comprise immediate, delayed and modified release preparations.

Corticosterone is an endogenous human glucocorticoid with similar and possibly enhanced binding affinity to GR and MR respectively, compared to cortisol (Mani et al. 2016). There is evidence for tissue-specific responses to cortisol and corticosterone depending on expression of ABC transporters. In the CNS, corticosterone is present in disproportionately high concentrations suggesting HPA negative feedback may be more sensitive to changes in corticosterone (Karssen et al. 2001; Raubenheimer et al. 2006). In metabolic tissues such as adipose tissue and skeletal muscle, ABCC1 actively exports corticosterone but not cortisol and may act in a protective manner to limit adverse metabolic effects of corticosterone (Nixon et al. 2016). We hypothesise that corticosterone would be a superior glucocorticoid than hydrocortisone for replacement therapy due to a reduction in adverse metabolic effects. Concerns regarding the effect of sustained release cortisol on bone mineral density may also be mitigated by the relatively high expression of ABCC1 in bones as well as adipose tissue and skeletal muscle.

This thesis aims to characterise the physiological differences between cortisol and corticosterone and define the role of ABCC1 in adipose tissue and skeletal muscle. We will also investigate the short-term outcomes in direct comparison of corticosterone and cortisol replacement in patients with CAH.

1.6 Hypotheses

- Corticosterone concentrations in plasma, saliva and hair do not simply mimic those of cortisol in normal physiology.

- In adipose tissue and skeletal muscle, cortisol is the principal glucocorticoid acting on GR and MR due to export of corticosterone by ABCC1.

- In congenital adrenal hyperplasia, glucocorticoid replacement with corticosterone results in less adverse metabolic sequelae than cortisol for equally effective suppression of ACTH.

1.7 Aims

1. To determine the characteristics of corticosterone concentrations in plasma, saliva and hair compared with cortisol in healthy individuals

2. To determine whether cortisol and corticosterone are differentially bound to GR and/or MR in adipose tissue and skeletal muscle

3. To determine whether the ABCC1 transporter is responsible for differential binding of cortisol and corticosterone to GR/MR in adipose tissue and skeletal muscle

4. To determine whether corticosterone suppresses ACTH and adrenal androgen production to a similar degree as cortisol in congenital adrenal hyperplasia

5. To compare the metabolic effects of corticosterone and cortisol replacement therapy in congenital adrenal hyperplasia

Chapter 2. Methods

2.1 Equipment

2.1.1 Laboratory-based

2.1.1.1 β-scintillation counter

- Berthold LB509 detector (Berthold, Hertfordshire, UK).

2.1.1.2 Balance

- Mettler HK 60 microbalance (Mettler Instrumente Ag, Zürich, Switzerland).

2.1.1.3 Centrifuge

- General Laboratory use: Eppendorf centrifuge 5810R (Cambridge, UK).
- Clinical sample processing (Chapter 3, 4 and 5): Sigma laboratory centrifuge 4K15 (Osterode am Harz, Germany).
- Real Time qPCR (Chapter 4): Eppendorf centrifuge 5415R (Cambridge, UK).

2.1.1.4 Incubator

- Grant-bio PHMP-4 Thermoshaker (Grant Instruments, Cambridge, UK).

2.1.1.5 Liquid chromatography

- Shimadzu Nexera LC-30AD ultra high performance liquid chromatograph (UHPLC) pump with Nexera SIL-30 AC autosampler (Shimadzu, Kyoto, Japan).

2.1.1.6 Mass spectrometer

- Linear ion trap QTRAP® 6500 triple quadrupole mass spectrometer configured with an ESI Turbo V source (Ab Sciex, Framingham, MA, USA).

2.1.1.7 Microplate shaker

- GFL Orbital Shaker 3005 (GFL, Burgwedel, Germany).

2.1.1.8 Microplate reader

- Optimax tuneable microplate reader (Molecular Devices, Sunnyvale, Ca).

2.1.1.9 Nitrogen dry-block

- Dri-Block® DB3A sample concentrator (Techne, Staffordshire, UK).
- Argonaut SPE Dry 96 Dual (Biotage, Uppsala, Sweden).

2.1.1.10 Real-Time PCR System

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

2.1.1.11 Spectrophotometer

- Nanodrop Spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA).

2.1.1.12 Thermal cycler

- TC-512 Gradient Thermal Cycler (Techne, Staffordshire, UK).

2.1.1.13 Vortex mixer

- Rotamixer (Hook and Tucker Instruments, Longfield, UK).

2.1.1.14 Water purification

- Milli-Q® Advantage A10 Water Purification System (Merck Millipore Corporation, Darmstadt, Germany).

2.1.1.15 96-well vacuum manifold

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

2.1.2 Clinical-based

2.1.2.1 Bioelectrical impedence

- OMRON BF302, OMRON Healthcare (UK) Ltd, Henfield, UK

2.1.2.2 Blood pressure and pulse measurement

- OMRON 705IT BP monitor, OMRON Healthcare (UK) Ltd, Henfield, UK

2.1.2.3 Electronic scales

- SECA 704, SECA Electronic Scales, Hamburg, Germany

2.1.2.4 Gamma counter

- GMS411 Mediscint gamma counter (John Caunt Scientific Ltd, Bury, UK)
- GMS411 Data Manager Version 12.0

2.1.2.5 Warm air box

- Manufactured in-house, calibrated to 60°C

2.1.2.6 Pulse wave analysis

- SphygmoCor System (AtCor Medical, Inc, USA) using AtCor Medical SCOR-2000 software, version 7.0.

2.1.2.7 Plethysmography

- AG101 Cuff Inflator source (Hokanson, WA, USA)

- E20 Rapid Cuff inflator (Hokanson, WA, USA)
- EC4 plethysmograph (Hokanson, WA, USA)
- Data displayed and analysed using Powerlab 4120 and LabChart® Reader (version 8) (AD Instruments, Oxford, UK)

2.1.2.8 Red light probe

- KL2500 LCD (Schott UK Ltd, Stafford, UK)

2.2 Materials

2.2.1 Radioactive substances

- ^{133}Xenon gas (USP grade: >99.5% purity) (Nordion, Ottawa, Canada).

  Specific activity 0.00667 mCi per μL

2.2.2 Solutions and solvents for clinical studies

- Saline (NaCl):

  Sodium chloride (0.9% weight/volume(w/v)) (Baxter, Newbury, UK), containing 77 mmol/500 ml (154 mM) sodium and 77 mmol/500 ml (154 mM) chloride

- Dextrose:

  Glucose 20% w/v containing 20g glucose per 100ml

- 0.1% (v/v) Diethylpyrocarbonate (DEPC) water:

  DEPC (1mL) was added to distilled water (1L). This was mixed and allowed to stand (room temperature) for 24 hours before autoclaving. Storage was at room temperature.

2.2.3 Drugs for clinical studies

2.2.3.1 Stable isotopically labelled tracers

2.2.3.1.1 D2-cortisone

1,2-$[^2H]_2$-cortisone; >96.3% purity and 98% isotopic enrichment as determined by thin layer chromatography (Cambridge Isotope Laboratories, Andover, MA, USA).

2.2.3.1.2 D8-corticosterone

2,2,4,6,6,17α,21,21-$[^2H]_8$-corticosterone; ≥98% purity and 98.5% isotopic enrichment as determined by thin layer chromatography (Cambridge Isotope Laboratories, Andover, MA, USA).

2.2.3.1.3 D2-glucose

6,6-$[^2H]_2$-glucose; 99.3% purity and 99% isotopic enrichment as determined by thin layer chromatography (Euriso-top, Saint-Aubin, France)

2.2.3.1.4 D5-glycerol

1,1,2,3,3-$[^2H]_5$-glycerol; 99.5% purity and >99% isotopic enrichment as determined by thin layer chromatography (Euriso-top, Saint-Aubin, France)

2.2.3.2 Unlabelled steroids

2.2.3.2.1 Hydrocortisone

Hydrocortisone sodium phosphate (Concordia, Ontario, Canada), 100mg/1mL.

2.2.3.3 Other drugs

2.2.3.3.1 Potassium canrenoate

Aldactone (Boehringer Ingelheim, Ingelheim am Rhein, Germany), 200 mg/10mL.

2.2.3.3.2 Mifepristone

Mifegyne (Exelgyn, Paris, France), 200mg tablets

2.2.3.3.3 Probenecid capsules

Probenecid (Arena Pharmaceuticals Ltd, Buckingham, UK), 500mg capsules.

2.3 Quantitation of glucocorticoids in plasma, saliva and hair

2.3.1 Materials and reagents

2.3.1.1 Reagents

High performance liquid chromatography (HPLC) grade water and formic acid were from Fisher Scientific (Loughborough, UK). HPLC grade methanol was from VWR (Lutterworth, Leicestershire, UK). Acetonitrile, ethyl acetate and chloroform were obtained from Rathburn chemicals Ltd. (Walkerburn, Scottish Borders, UK).

2.3.1.2 Glucocorticoids

Unlabelled steroids cortisol (F), corticosterone (B), epi-corticosterone (epi-B), cortisone (E) and 11-dehydrocorticosterone (A) were supplied by Sigma Aldrich (St. Louis, MO, USA). Deuterium-labelled steroids deuterated cortisol (9,11,12,12-$[^2H]_4$-cortisol, D4-cortisol), deuterated cortisone (1,2-$[^2H]_2$-cortisone, D2-cortisone) and deuterated corticosterone (2,2,4,6,6,17α,21,21-$[^2H]_8$-corticosterone, D8-corticosterone) were supplied by Cambridge Isotopes (Tewksbury, MA, USA). Each of these steroids were weighed and dissolved in methanol (final concentration 1 mg/mL) and stored at -20 °C.

2.3.1.3 Other analytes

Mifepristone (RU486), canrenone, alfaxolone and deuterated canrenone (D6-canrenone) were supplied by Sigma Aldrich (St. Louis, MO, USA).

2.3.2 Preparation of standard curves

Stock solutions of the individual glucocorticoids (1 mg/mL) were diluted in methanol and mixed for serial dilution. Aliquots were pipetted into glass tubes producing a concentration range as specified in each section. Internal standards for each analyte were added. The volume of each standard was made up to 400 µL with water. The standards were then extracted according to the extraction protocol for the biological fluid of interest.

2.3.3 Extraction of glucocorticoids from plasma

Plasma samples were defrosted at room temperature. Samples (200 µL) enriched with internal standard (specified in the relevant section) were extracted by supported liquid extraction (SLE). Enriched samples were added to a disposable glass tube along with 190 µL HPLC grade water and mixed thoroughly using a vortex. Samples were then transferred to an SLE 400+ 96-well plate (Biotage, Uppsala, Sweden) using a glass Pasteur pipette. The plate was placed in a 96-well vacuum manifold and a vacuum was applied for 5 minutes. Samples were eluted with 0.9 mL of dichloromethane and isopropanol (98:2) twice into a 96-well collection plate (Waters, Hertfordshire, UK). Each sample was allowed to elute under gravity for 5 minutes then the vacuum was applied for 2 minutes. Samples were dried down under nitrogen at 60 °C using a 96 well nitrogen dry block (Biotage, Uppsala, Sweden) and stored at -20 °C until analysis. Prior to analysis, samples were re-suspended in HPLC grade water and acetonitrile (70:30, 70 µL) and mixed on a plate shaker.

2.3.4 Extraction of glucocorticoids from saliva

The extraction method was optimised for extraction efficiency and ion suppression of internal standards by comparing unextracted and extracted samples with 3 replicates (Table 2-1).

Table 2-1: Extraction efficiency and ion suppression of internal standards

Internal Standard	**% Recovery**	**% Ion Suppression**
D4 Cortisol	78.92	77.18
epi Corticosterone	82.99	80.53

Intra-assay analysis was assessed using 6 replicates at low (0.005 ng), medium (0.01 ng) and high (0.025 ng) concentration. Lower limit of quantitation (LLOQ) was determined when relative standard deviation (%RSD) was 20% or less. Accuracy was accepted when less than 10 (Table 2-2).

Saliva samples (200 μL) enriched with internal standard (D4-F, epi-B, 10 ng of each) were extracted by supported liquid extraction. Enriched samples were added to a disposable glass tube along with 200 μL zinc sulphate and were subject to centrifugation (10 minutes, 32 869 g, 4 °C). Samples were then transferred to SLE 400+ 96-well plate (Biotage, Uppsala, Sweden) using a glass Pasteur pipette. The plate was placed in a 96-well vacuum manifold and a vacuum was applied for 5 minutes.

Samples were eluted with 0.9 mL of dichloromethane and isopropanol (98:2) twice into a 96 well collection plate (Waters, Hertfordshire, UK).

Table 2-2: Limits of quantitation and expected ranges (400 μL saliva)

LLOQ: Lower limit of quantitation; %RSD: % relative standard deviation.

Analyte	**Expected range in saliva (am)**		**Results from 6 replicate extracts**		
	(nmol/L)	(ng/mL)	LLOQ (ng)	%RSD	Accuracy
11-dehydrocorticosterone	No clinical data	No clinical data	0.025	20.34	0.09
Corticosterone	No clinical data	No clinical data	0.005	15.72	6.67
Cortisone	5-40*	4-20	0.025	13.8	0.17
Cortisol	2-15*	1-5	0.025	20.03	3.33

* (Perogamvros et al. 2009; Perogamvros, Keevil, et al. 2010; Gao et al. 2015)

Each sample was allowed to elute under gravity for 5 minutes then the vacuum was applied for 2 minutes. Samples were dried down under nitrogen at 60 °C using a 96 well nitrogen dry block (Biotage, Uppsala, Sweden). A further identical extraction was carried out using another 200 μL of saliva sample and added to the initial sample on re-suspension.

Samples were stored at -20 °C until analysis. Prior to analysis, samples were re-suspended in HPLC grade water and acetonitrile (70:30, 70 μL) and mixed on a plate shaker.

2.3.5 Extraction of glucocorticoids from hair

Hair samples were washed gently with 2 mL LC-MS grade isopropanol at room temperature and left to dry in the fume hood. Each segment was weighed and transferred to a fresh glass tube. 1.4 mL LC-MS grade methanol was added to each tube along with internal standard (1 ng). Tubes were incubated at 25 °C for 18 hours. Samples were subject to centrifugation at 4302 g for 5 minutes at 4 °C. 1 mL of the clear supernatant from each sample was transferred to fresh glass tube and dried down under nitrogen at 50 °C. The samples were re-suspended in 1 mL of 2% HPLC grade methanol and vortexed for 1 minute. Hair extracts were then cleaned by solid phase extraction using Oasis HLB 10 μm cartridges (Waters, MA, USA). Cartridges were conditioned with 1 mL of methanol followed by 1 mL of HPLC grade water. Samples were loaded (1 mL) into the cartridges, washed with 1 mL 30% methanol and eluted twice with 300 μL methanol. Extracts were dried down under nitrogen at 50 °C and stored at 4 °C until analysis.

2.3.6 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Two methods were used for LC-MS/MS analysis in this thesis. In chapters 3 and 4, the glucocorticoid assay was used and in chapter 5, the combined glucocorticoid and androgen assay was used.

2.3.6.1 Instrument

The Shimadzu liquid chromatograph coupled with a QTRAP® 6500 mass spectrometer (Ab Sciex, Framingham, MA, USA) was used for all experiments.

2.3.6.2 Glucocorticoid assay

2.3.6.2.1 Chromatography conditions

Steroids were separated on an ACE Excel C18 AR column (150 x 2.1 mm, 2 μm) at 40 °C, at a flow rate of 5 mL/min with a total run time of 9 minutes. A gradient solvent system was used as shown in Table 2-3.

Table 2-3: Solvent gradient

Time (mins)	Water with 0.1% formic acid (FA)	Acetonitrile with 0.1% FA
0.01	70	30
4	70	30
6	10	90
7	10	90
7.1	70	30
9	Stop	

2.3.6.2.2 Mass spectrometry conditions

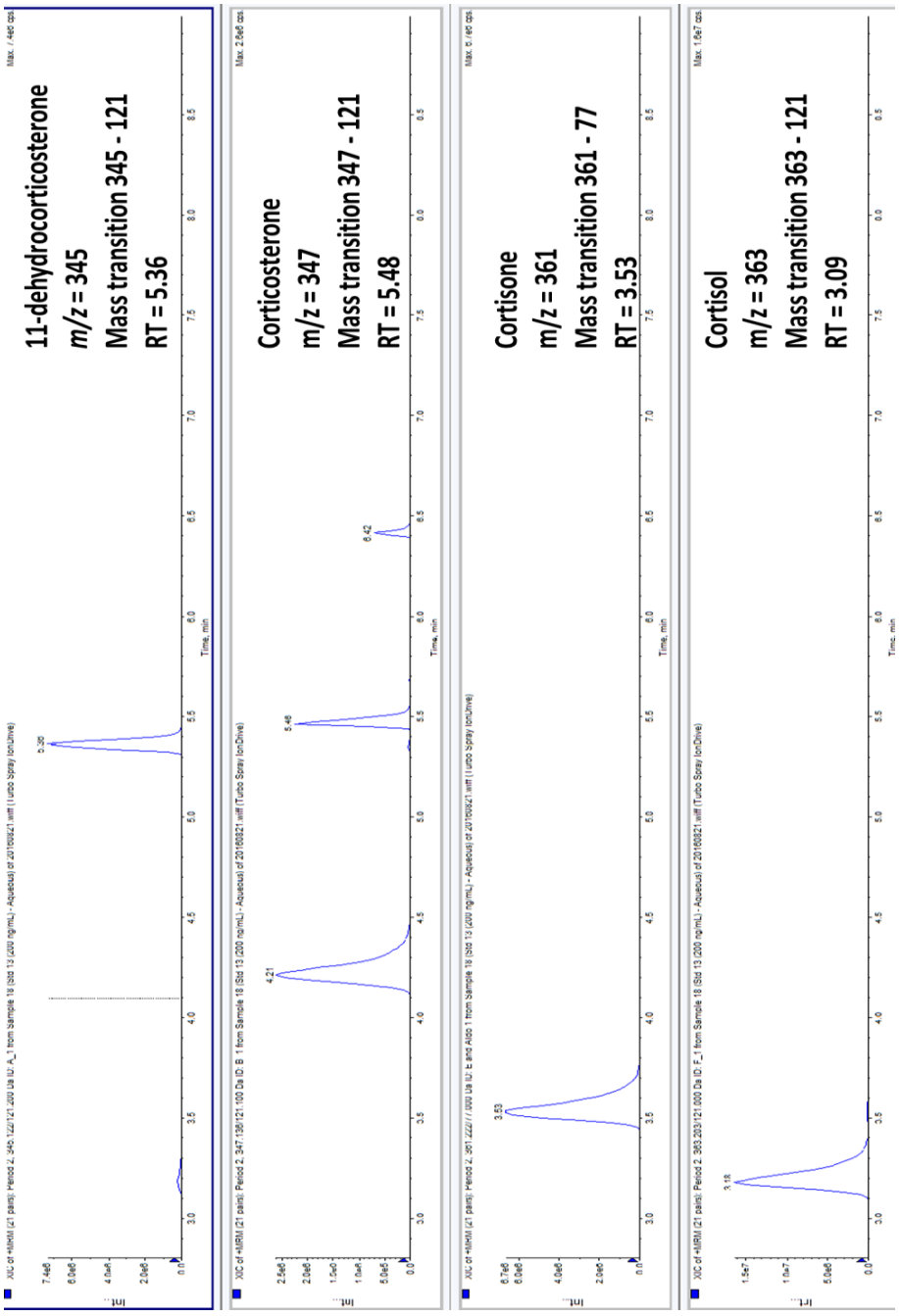
Ionisation was performed in positive electrospray mode with curtain gas 40 psi, collision gas medium, spray voltage 5500 V, source temperature 700 °C, source gases 40 psi. Mass transitions, retention times and collision energies of the analytes are displayed in Table 2-4. Typical chromatography is shown in Figure 2-1.

Table 2-4: Chromatographic conditions for LC-MS/MS

Analyte	**Mass transition of protonated ion (m/z)**	**Retention Time (mins)**	**De-clustering potential (V)**	**Collision Energy (V)**	**Collision Cell Exit Potential (V)**
Cortisol	363.1→121.1	3.09	141	101	14
Cortisone	361.2→77.2	3.53	166	99	36
Corticosterone	347.1→121.1	5.48	66	69	8
11-dehydrocorticosterone	345.1→121.1	5.36	57	33	8
D4-cortisol	366.9→121	3.46	166	41	54
Epi-corticosterone	347.1→121	4.07	66	69	8

Figure 2-1: Typical chromatography for glucocorticoid assay

m/z: mass to charge ratio; RT: retention time



2.3.6.3 Glucocorticoid and androgen method

2.3.6.3.1 Chromatography conditions

Steroids were separated on a Kinetex C18 column (150 x 3 mm, 2.6 μm) at 40 °C, at a flow rate of 5 mL/min with a total run time of 14 minutes. A gradient solvent system was used as shown in Table 2-5.

Table 2-5: Solvent gradient

Time (mins)	**Water with 0.1% FA (%)**	**MeOH with 0.1% FA (%)**
0.01	45	55
4	45	55
10	0	100
12	0	100
12.1	45	55
14	Stop	

2.3.6.3.2 Mass spectrometry conditions

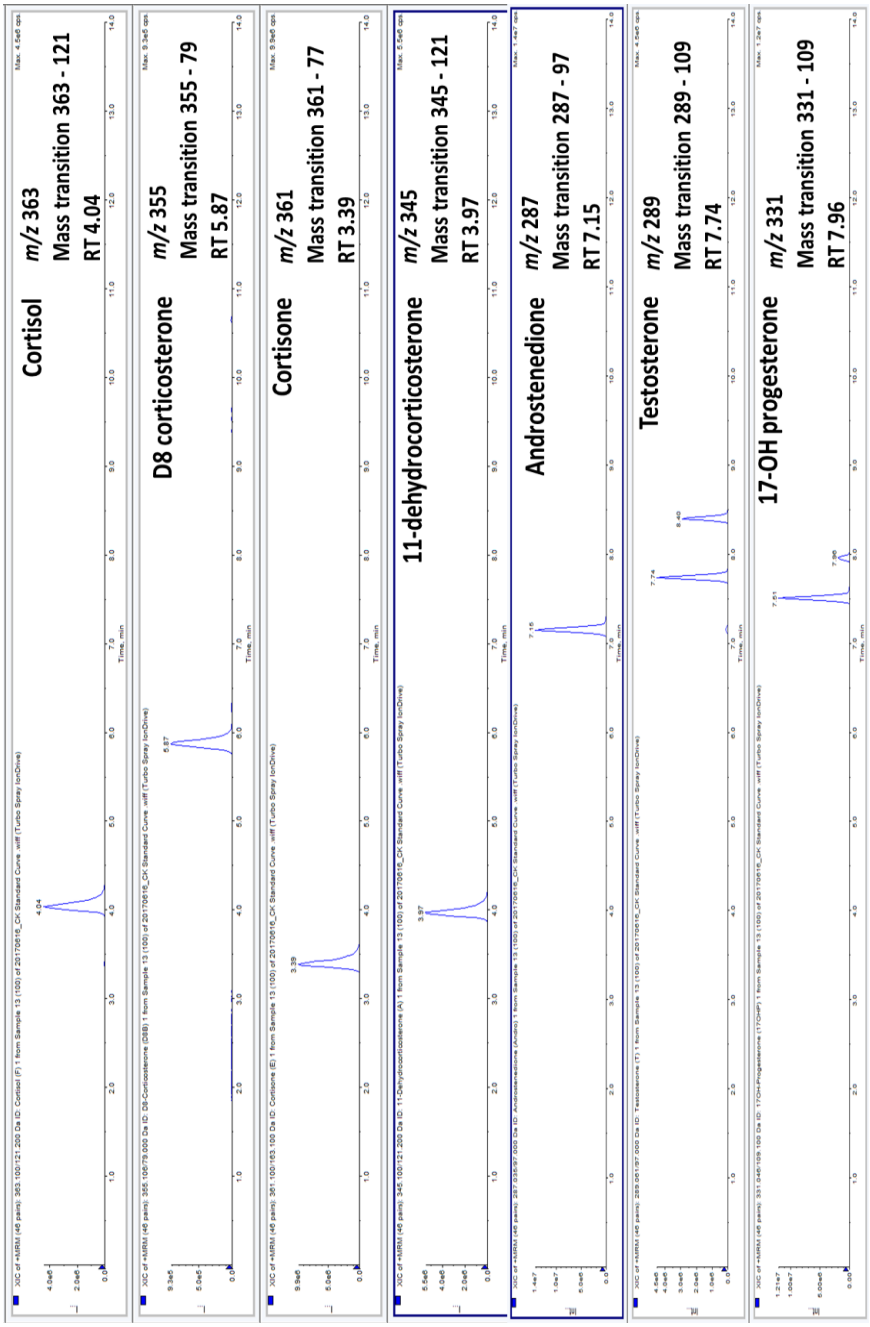
Ionisation was performed in positive electrospray mode with curtain gas 40 psi, collision gas medium, spray voltage 5500 V, source temperature 500 °C and source gases 40 psi. Typical chromatography is shown in Figure 2-2.

Table 2-6: Chromatographic conditions for LC-MS/MS

Analyte	**Mass transition of protonated ion (m/z)**	**Retention Time (mins)**	**De-clustering potential (V)**	**Collision Energy (V)**	**Collision Cell Exit Potential (V)**
Cortisol	363.1→121.1	4.04	141	101	14
Cortisone	361.2→77.2	3.39	166	99	36
D8-corticosterone	355.1→79	5.87	56	97	12
Corticosterone	347.1→121.1	5.41	66	69	8
11-dehydrocorticosterone	345.1→121.1	3.97	57	33	8
Testosterone	289.1→109.2	7.74	101	31	6
Androstenedione	287→97	7.15	61	27	14
17-OH progesterone	331→109.1	7.96	66	31	12
D4-cortisol	366.9→121	3.99	166	41	54
Epi-corticosterone	347.1→121	4.42	66	69	8

Figure 2-2: Typical chromatography for glucocorticoid and androgen assay

m/z: mass to charge ratio; RT: retention time



2.3.7 Data analysis

MultiQuant® software was utilised to integrate area under the peak for each analyte and internal standard. The ratio of analyte peak area against its corresponding internal standard was used for quantification against a standard curve. A line of best fit (y = mx + c) was drawn after plotting known steroid concentrations (x axis) against corresponding analyte to internal standard peak area ratio (y axis) with a weighting of (1/x). A regression coefficient (r^2) of >0.9 was accepted for each standard curve.

2.4 Quantitation of salivary corticosterone by enzyme-linked immunosorbent assay (ELISA)

Salivary corticosterone was measured using enzyme linked immunosorbent assay (ELISA) (Enzo Life Sciences, Exeter, UK). All reagents were stored at 2-8°C and allowed to come to room temperature before each assay. Assay and wash buffers were prepared with deionised water at 1:10 and 1:100 dilutions respectively. Standards were prepared by serial dilution to 20000, 4000, 800, 160 and 32 pg/ml concentrations and used within 60 minutes of preparation. Samples were extracted before analysis using liquid-liquid extraction with ethyl acetate. 100 µL of saliva was added to 400 µL of deionised water which was extracted twice with 4 mL of ethyl acetate before drying down with nitrogen at 60 °C. Samples were reconstituted using the sample diluent provided. Samples and standards (200µl) were added to a 96-well plate coated with donkey antibody specific to sheep IgG. Alkaline phosphatase conjugated with corticosterone (50µl) was added to each well along with 50µl of a sheep polyclonal

antibody to corticosterone. The plate was incubated at room temperature on a plate shaker for 2 hours. The contents of the wells were emptied and washed 3 times with wash buffer. After the final wash, 200µl of substrate p-nitrophenyl phosphate buffer solution was added to each well and left for 1 hour at room temperature without shaking. The enzyme reaction was stopped with 50µl of stop solution and the absorbance was read (405 nm; correction between 570 and 590nm) in a spectrophotometric microtiter plate reader. A standard curve was generated by plotting absorbance against corticosterone concentration for each standard by fitting to a 4-parameter curve (SoftMax Pro, Sunnyvale, Ca). Sensitivity was determined at 26.99 pg/mL. Intra-assay precision for low, medium and high concentration with n=16 was determined with %CV 8.0, 8.4 and 6.6% respectively. Inter-assay precision was measured in low, medium and high concentration with n=8 with %CV 13.1, 8.2 and 7.8% respectively. Samples were measured in duplicate and accepted if %CV was <15%.

2.5 Quantitation of plasma ACTH by ELISA

Plasma ACTH was measured using a two-site ELISA (Biomerica, Irvine, California). All reagents were stored at 2-8°C and allowed to come to room temperature before each assay. Assay calibrators and controls were reconstituted with deionised water (2 mL) and mixed gently. Reconstituted calibrators and controls were stored (-20°C) for 6 weeks with up to 3 freeze thaw cycles. Wash buffer concentrate (30 mL) was diluted 1:20 in deionised water. The diluted wash buffer was stable for up to 90 days at room temperature.

For each patient, samples from both study visits were assayed together. Assay standards (0-541 pg/ml), controls and samples (200 μL) were added in duplicate to a 96-well microtitre plate coated with streptavidin. Biotinylated ACTH antibody (25 μL) was added to each well followed by horseradish peroxidase (HRP) enzyme labelled ACTH antibody (25 μL). The plate was covered in aluminium foil to avoid light exposure and placed on a plate shaker for incubation (at room temperature for 4 hours). Unbound antibodies and buffer matrix were then removed by washing 5 times with wash solution (350 μL). For the detection of the immunocomplex, tetramethylbenzidine (TMB) substrate solution (150 μL) was added to each well and incubated for a further 30 minutes on a plate shaker (covered with aluminium foil). The HRP/TMB reaction was terminated with acidic stopping solution (100μL) and mixed gently. The absorbance was read within 10 minutes in a spectrophotometric microtitre plate reader at both 405 and 450nm. A standard curve was generated by plotting absorbance against plasma ACTH concentration for each calibrator by fitting to a 4-parameter curve (SoftMax Pro, Sunnyvale, Ca).

Intra-assay CV, as assessed by measurement of ACTH concentration at low and high concentration in n=25 assays was 6.71% at 42.2 pmol/L and 2.27% at 269.9 pmol/L. Inter-assay CV was determined from two samples on 21 different days and was 7.1% and 6.9% at 42.3 pmol/L and 287.8 pmol/L respectively.

2.6 Quantitation of serum insulin by ELISA

Serum insulin was measured using a solid phase two-site enzyme immunoassay (DRG Diagnostics, Marburg, Germany). All reagents were stored at 2-8 °C and allowed to

come to room temperature before each assay. Wash buffer concentrate (50 mL) was diluted in 1000 mL deionised water. Peroxidase conjugated mouse monoclonal anti-insulin antibody was diluted 1:11 in supplied diluent. Standards containing recombinant human insulin (0-20 mU/L) and samples (25 μL) were added in duplicate to wells of a microtiter plate coated with mouse monoclonal anti-insulin antibody. Diluted peroxidase antibody (100 μL) was added. The plate was sealed, covered and incubated on a plate shaker (900 rpm) for 1 hour at room temperature. Unbound enzyme labelled antibody and buffer matrix were removed by washing 6 times with dilute wash solution (350 μL). TMB substrate solution (200 μL) was added to each well and the plate sealed, covered and incubated for 30 minutes at room temperature. TMB stop solution (0.5M H_2SO_4, 50 μL) was added and mixed before measuring absorbance at 450 nm in a spectrophotometric microtiter plate reader. A standard curve was generated by plotting absorbance against serum insulin concentration for each standard and using cubic spline regression (SoftMax Pro, Sunnyvale, Ca). Intra-assay precision was determined with 6 replicates for low, medium and high concentration with %CV 5.3, 4.2 and 5.1% respectively. Inter-assay precision for the same concentrations had %CV of 2.7, 3.9 and 1.8% respectively. The limit of detection was 0.15 mU/L.

2.7 Quantitation of serum non-esterified fatty acids by enzymatic colorimetric assay

Serum non-esterified fatty acids (NEFAs) were quantified by colorimetric assay (Wako Chemicals, Germany). Standards (0-1000 μM) were prepared by serial dilution

in deionized water and stored at 4 °C. Standards and samples (10 μL) were added to the wells of a 96-well plate, cooled on ice. Reconstituted reagent A (160 μL), containing acyl-CoA synthetase, was added to each well. The plate was incubated (37 °C, 5 minutes) and mixed gently while NEFAs were converted to fatty acyl-CoA thiol esters, which react with oxygen in the presence of acyl-CoA oxidase to produce hydrogen peroxide. The optical density of each well was measured at 550 nm and 660 nm to obtain a blank reading to allow for colour differences in the plasma samples. Reconstituted reagent B (80 μL), containing acyl-CoA and peroxidase, was then added to each well, allowing the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline with 4-aminoantipyrine to form a purple pigment. The plate was incubated again (37 °C, 5 minutes) with gentle mixing. The optical density of each well was again measured at 550 nm and 660 nm to give an actual reading. For both blank and actual reading, 660 nm was subtracted from 550 nm before blank was subtracted from actual readings. Serum NEFA concentration was calculated from a standard curve generated by linear regression analysis of absorbance plotted against NEFA concentration in standards. Precision was determined for 5 replicates at <1.5%.

2.8 Quantification of mRNA abundance in subcutaneous adipose tissue

2.8.1 Materials and reagents

RNA was extracted from adipose tissue using QIAGEN® RNeasy Mini Kit (QIAGEN®, Hilden, Germany). RLT, RPE and RW1 buffers were provided in the kit. Qiazol was obtained separately from QIAGEN® (Hilden, Germany).

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

2.8.2 Tissue collection

A biopsy of subcutaneous adipose tissue was taken with informed written consent, as per the clinical study protocols (Chapter 4.2.5 and Chapter 5.2.5). Samples were cleaned with DEPC water and frozen immediately on dry ice and stored at -80 °C until analysis.

2.8.3 RNA extraction from subcutaneous adipose

Adipose tissue (~100 mg) was homogenised in Qiazol Lysis Reagent (1 mL, Qiagen) using ball bearings and a shaker. Chloroform (200 μL) was added, mixed and subject to centrifugation (12, 000 g for 15 minutes at 4 °C). The supernatant (approximately 600 μL) was transferred to a 1.5 mL Eppendorf and an equal volume of ethanol (70%, *v/v*) was added and mixed. The solution was transferred to an RNase spin column and

the eluate discarded after centrifugation (12,000 g for 30 seconds at room temperature). The column was washed with Buffer RW1 (700 μL) and Buffer RPE (500 μL) sequentially and eluate was discarded after centrifugation (12,000 g for 30 seconds at room temperature). A further wash of the membrane with Buffer RPE (500 μL) was subject to centrifugation again (12,000 g for 2 minutes at room temperature). The RNeasy spin column was placed in a fresh collection tube and subject to centrifugation until dry (12,000 g for 1 minute at room temperature). The RNase spin column was placed in a fresh Eppendorf (1.5 mL). RNase-free water (30 μL) was added to the spin column, incubated for 1 minute and eluted by centrifugation (12,000 g for 1 minute at room temperature). The eluate was then added back to the RNeasy spin column, incubated for 1 minute and subject to centrifugation again (12,000 g for 1 minute at room temperature). RNA was stored at -80°C.

2.8.4 RNA quantification

RNA was quantified using a Nanodrop Spectrophotometer (Thermo Fisher, West Sussex, UK). Concentration was determined by the absorbance at 260 nm wavelength and the purity assessed by the ratio of RNA/DNA (260/280), which was deemed acceptable if between 1.8 and 2.0.

2.8.5 RNA quality

RNA quality was assessed by electrophoresis on a denaturing agarose gel (1.2% *w/v* in 1 x TBE). Samples (2 μL) were prepared by adding loading dye (Promega, WI, USA; 1 in 5 dilution in RNase free water; 10 μL). Prepared samples were electrophoresed on the gel (100 V, 45 minutes) and RNA integrity was deemed

satisfactory if clear 28S and 18S bands were present without smearing, and if the 28S rRNA band was approximately twice the intensity of the 18S rRNA band.

2.8.6 Reverse transcription polymerase chain reaction

RNA was reverse transcribed using the Quantitect Reverse Transcription kit (Qiagen, UK). 500 ng of RNA was used and made up to 12 μL with RNase free water. RNA was added to genomic DNA (gDNA) wipeout buffer (2 μL) and incubated (at 42 °C for 2 minutes) to eliminate any contaminating gDNA. Quantiscript Reverse Transcriptase (1 μL), Quantiscript RT buffer (4 μL) and RT primer mix (1 μL) were added to each sample. A negative control was prepared as above except replacing reverse transcriptase with water (denoted "–RT control"). Samples were incubated (at 42 °C for 15 minutes, then 95 °C for 3 minutes) in PCR thermal cycler, before being cooled to 4°C. Resultant cDNA was stored at -20 °C.

2.8.7 Real-time polymerase chain reaction (RT-PCR)

2.8.7.1 Materials and reagents

Primers were obtained from Invitrogen Life Technologies (Thermo Fisher Scientific Co, Waltham, MA, USA). Universal Probe Library (UPL®) Probes, Probe Mastermix and Lightcycler RNase free water were obtained from Roche Diagnostics Ltd (Burgess Hill, UK). Primers were designed to match intro-spanning probes within the UPL using online software (Universal Probe Library Assay Design Centre).

2.8.7.2 RT-PCR

For each gene, a standard curve was made up from pooling all samples and prepared by serial dilution in RNase free water at concentrations of: 1:8; 1:16; 1:32; 1:64; 1:128; 1:256; 1:512. cDNA samples were diluted (1:10) with RNase free water. A Mastermix of UPL Fastmix (5 μL), RNase free water (2.7 μL), forward primer (0.1 μL), reverse primer (0.1 μL) and probe (0.1 μL) was prepared for each sample. One gene was analysed with SYBR green master mix (Table 2-8) where mastermix (5 μL), RNase free water (2 μL), forward primer (0.5 μL) and reverse primer (0.5 μL) were prepared for each sample. A –RT control was analysed with each gene. Diluted cDNA (2 μL) was added to each well along with 8 μL of master mix. All samples and standards were assessed in triplicate. The plate was covered and subject to centrifugation (1500 g for 2 minutes) before being run on the Light Cycler.

Samples were denatured by heating (95 °C, 5 minutes), then amplified for 50 cycles of consisting of denaturation (10 secs, 95 °C), annealing (30 secs, 60 °C) and elongation (1 sec, 72 °C) and cooling (30 secs, 40 °C).

All samples were analysed in triplicate. Amplification curves were plotted for each sample (y = fluorescence, x = cycle number). Triplicates were deemed acceptable if the standard deviation of the crossing point was <0.3 cycles. The standard curve generated for each gene (y = crossing point, x = log concentration) was deemed acceptable if the reaction efficiency was between 1.7 and 2.1.

2.8.7.3 Data analysis

The abundance of each gene was expressed relative to the mean of housekeeping genes (18S and Cyclophillin A, Table 2-7) (e.g. abundance of gene of interest in sample X/mean abundance of 18S and Cyclophillin A in sample X) and expressed as arbitrary units. Tables 2-8 and 2-9 give details of the primers and UPL probes used.

Table 2-7: Housekeeping genes

Gene: Name Gene accession number	**Primer Sequence**		**UPL Probe**
PPIA: Peptidylprolyl isomerase A (cyclophilin A) NM_021130.3	Forward	ATGCTGGACCCAACACAAAT	48
	Reverse	TCTTTCACTTTGCCAAACACC	
RNA18S: ribosomal RNA 18s	Forward	CTTCCACAGGAGGCCTACAC	46
	Reverse	CGCAAAATATGCTGGAACTTT	

Table 2-8: Primer sequence for qPCR and corresponding probe number for genes of interest from Roche Universal Probe Library (UPL®).

Gene: Name Gene accession number	**Primer Sequence**		**UPL Probe**
ADIPOQ: Adiponectin NM_004797.3	Forward	GGTGAGAAGGGTGAGAAAGGA	85
	Reverse	TTCACCGATGTCTCCCTTAG	
PNPLA2: Adipose Triglyceride Lipase NM_020376.3	Forward	CTCCACCAACATCCACGAG	89
	Reverse	CCCTGCTTGCACATCTCTC	
PER1: Period Circadian NM_002616.2	Forward	CTCTTCCACAGCTCCCTCA	87
	Reverse	CTTTGGATCGGCAGTGGT	
LPL: Lipoprotein Lipase NM_000237.2	Forward	ATGTGGCCCGGTTTATCA	25
	Reverse	CTGTATCCCAAGAGATGGACATT	
PEPCK: Phosphoenolpyruvate carboxykinase NM_002591.3	Forward	CGAAAGCTCCCCAAGTACAA	20
	Reverse	GCTCTCTACTCGTGCCACATC	
ABHD5: Abhydrolase domain containing 5 NM_016006.5	Forward	GGACAAAATGATCTTGCTTGG	66
	Reverse	CCCAAGGCTCCACTAAAATG	
NR3C1: Nuclear receptor subfamily 3, group C, member 1 (α GR) NM_000176.2	Forward	TTTTCTTCAAAAGAGCAGTGGA	11
	Reverse	GCATGCTGGGCAGTTTTT	
NR3C2: Nuclear receptor subfamily 3, group C, member 2 (MR) NM_000901.4	Forward	CATCATGAAAGTTTTGCTGCTACT	64
	Reverse	TCTTTGATGTAATTTGTCCTCATTTC	
SGK1: Serum and glucocorticoid-regulated kinase 1 NM_001143676.1	Forward	GACAGGACTGTGGACTGGTG	24
	Reverse	TTTCAGCTGTGTTTCGGCTA	
FKBP5: FK506 binding protein 5 NM_001145775.2	Forward	GGATATACGCCAACATGTTCAA	15
	Reverse	CCATTGCTTTATTGGCCTCT	

Table 2-9: Primer sequence for qPCR using SYBR® Green master mix

Gene: Name **Gene accession number**	**Primer Sequence**	
LIPE: Hormone Sensitive Lipase Arner Seq 2 NM_005357.3	Forward	GGAAGTGCTATCGTCTCTGG
	Reverse	GGCAGTCAGTGGCATCTC

2.9 Routine laboratory tests

Routine laboratory blood tests for screening in clinical studies were sent for processing by the NHS Lothian clinical biochemistry and haematology laboratories at the Western General Hospital and Royal Infirmary of Edinburgh (Edinburgh, UK). The laboratory participates in the UK National External Quality Assessment Service to ensure quality control. Sample collection is described in Chapters 3, 4 and 5.

Full blood count was measured by a XE-5000 automated flow cytometer (Sysmex UK, Milton Keynes, UK). Thyroid function was measured using Architect i2000 immunoassay (Abbott Diagnostics Ltd, Maidenhead, UK). All other analytes were measured on an Architect c16000 analyser (Abbott Diagnostics Ltd, Maidenhead, UK) using manufacturer's kit materials according to laboratory protocols.

Chapter 3. Physiology of corticosterone in healthy individuals

3.1 Introduction

Cortisol is routinely measured in human biological samples and physiology in healthy individuals is well established. In contrast, corticosterone is not routinely measured and although physiology is assumed to be similar to cortisol, the evidence for this is limited.

Cortisol is released in a constant and reproducible circadian pattern characterised by a peak in the morning beginning prior to waking at 3am, peaking at 9am with a nadir in the evening around midnight (Selmaoui & Touitou 2003; Dickmeis 2009; Chan & Debono 2010). In plasma, cortisol circulates bound to CBG and albumin with 5-10% free and unbound (Lin et al. 2010). Clinically, adequacy of adrenal glucocorticoid secretion can be determined as part of a dynamic test measuring stimulated plasma cortisol concentrations (Wood et al. 1965; Bornstein et al. 2016). Plasma cortisol generally reflects total cortisol which includes both bound and un-bound portions. Free cortisol is seldom measured clinically as assays are time consuming and unsuitable for routine use (Turpeinen & Hämäläinen 2013).

Salivary cortisol is a useful non-invasive method of measuring cortisol. This reflects the free unbound portion of circulating plasma cortisol which freely diffuses across salivary ducts (Hawley & Keevil 2016). Measurements correlate well with circulating plasma values and late night salivary cortisol is a validated screening test for Cushing's syndrome (Vining et al. 1983; Nieman et al. 2008).

An alternative non-invasive method for monitoring cortisol is through scalp hair measurement. A number of studies have validated hair cortisol measurement, showing

a time line of cortisol exposure over several months (Kirschbaum et al. 2009; Manenschijn et al. 2011; D'Anna-Hernandez et al. 2011). Given the circadian and ultradian variations in cortisol, this technique is unique in reflecting long term exposures to cortisol rather than single point in time measurements.

As discussed previously, corticosterone has been measured in human clinical plasma samples from the early days of glucocorticoid research (Sweat 1955; Peterson 1957; Ely et al. 1958; Peterson & Pierce 1960). The assays in use at that time had poor specificity and more recent studies suggest plasma corticosterone circulates in concentrations approximately 10-20 fold lower than cortisol (Seckl et al. 1990; Karssen et al. 2001). Most studies measured early morning samples however some also measured day curves which suggest corticosterone too has a diurnal rhythm (Martin & Martin 1968; Hamanaka et al. 1970; West et al. 1973). Again, the assays used in these studies lacked specificity and there is a high chance of cross-reactivity.

Salivary corticosterone is less well studied and although paired plasma and saliva concentrations have been studied, correlation has not yet been established (McVie et al. 1979). As might be expected given the difference in circulating plasma concentrations, salivary corticosterone concentrations were lower than cortisol. Again, this study used radioimmunoassay which lacks the specificity of tandem mass spectrometry. A literature review revealed no published evidence of either salivary day curves of corticosterone in healthy individuals or measurements of corticosterone in hair.

ABC transporter expression in salivary glands is low compared to adipose tissue and brain (Nishimura & Naito 2005) however it is reported that ABCB1 is expressed more highly than ABCC1 (Uematsu et al. 2003). This could result in relative corticosterone excess in saliva, similar to the hypothesised excess in brain due to export of cortisol by ABCB1. In contrast, ABCC1 expression in hair follicles is reported to be higher than ABCB1 and might suggest corticosterone will be difficult to measure in hair (Haslam et al. 2013). Given the uncertainty of how glucocorticoids are incorporated into hair however, the exact location of ABCC1 expression within the hair follicle is significant and as yet undefined. If ABCC1 is expressed in luminal epithelial cells for example, this could lead to higher corticosterone concentrations in hair.

While historic data suggests corticosterone has a similar diurnal rhythm to cortisol in plasma, this has not been tested using a specific mass spectrometry assay and there is very limited data investigating corticosterone concentrations in saliva and none, to our knowledge, in hair. With differential ABC transporter expression resulting in tissue specific differences between cortisol and corticosterone, it cannot be assumed that corticosterone simply mimics cortisol within these biological fluids and tissues. Independent analysis of corticosterone using modern techniques is necessary to define the physiology of corticosterone in humans.

We hypothesised that corticosterone physiology differs from cortisol in humans and that salivary and hair corticosterone concentrations are not directly comparable to cortisol. This chapter aimed to measure and explore the relationship between corticosterone and cortisol in human plasma, saliva and hair in healthy individuals.

3.2 Hair corticosterone

Corticosterone has never to our knowledge been measured in hair and we aimed to achieve this by adapting a validated LC-MS/MS technique for cortisol (Noppe et al. 2015).

3.2.1 Methods

3.2.1.1 Hair sample preparation

A hair sample was obtained from one healthy female volunteer (age 31, BMI 22). Approximately 100-200 hairs were cut from the posterior vertex, as close to the scalp as possible.

The initial analysis used the proximal 5 cm of hair, reflecting the most recent 5 months. The sample was cut into 1 cm segments each weighing 10-25 mg and transferred to disposable glass tubes.

A further assay was performed with the remaining hair sample analysed as one sample weighing 247 mg.

3.2.1.2 Preparation of standard curve

Standard curves were prepared for analytes in Table 3-1 as described in section 2.3.2.

Table 3-1: Standard curve analytes and internal standards for hair

Analyte	Standard Curve Range (ng)	Internal Standard	Internal Standard amount (ng)
Cortisol	0.01-5	D4-cortisol	1
Cortisone	0.01-5	D4-cortisol	1
Corticosterone	0.01-5	Epi-corticosterone	1
11-dehydrocorticosterone	0.01-5	Epi-corticosterone	1

3.2.1.3 Extraction of samples

Samples were extracted using method described in section 2.3.5.

3.2.1.4 LC-MS/MS analysis

Samples were analysed using the method described in section 2.3.6.2.

3.2.2 Results

3.2.2.1 Glucocorticoid concentrations in 5 hair samples

Cortisol and cortisone were detected in all 5 samples at varying concentrations (Figure 3-1). Corticosterone and 11-dehydrocorticosterone were not detectable in any of the samples.

3.2.2.2 Glucocorticoid measurements in large hair sample

Cortisol, corticosterone and their inactive metabolites cortisone and 11-dehydrocorticosterone were detectable in this sample and are shown in Figure 3-2. The ratio of corticosterone: cortisol was 0.025.

Figure 3-1: Hair cortisol and cortisone concentrations in one individual

Data are for individual samples over five consecutive months for hair cortisol (blue fill) and cortisone (purple fill) concentrations in one individual.

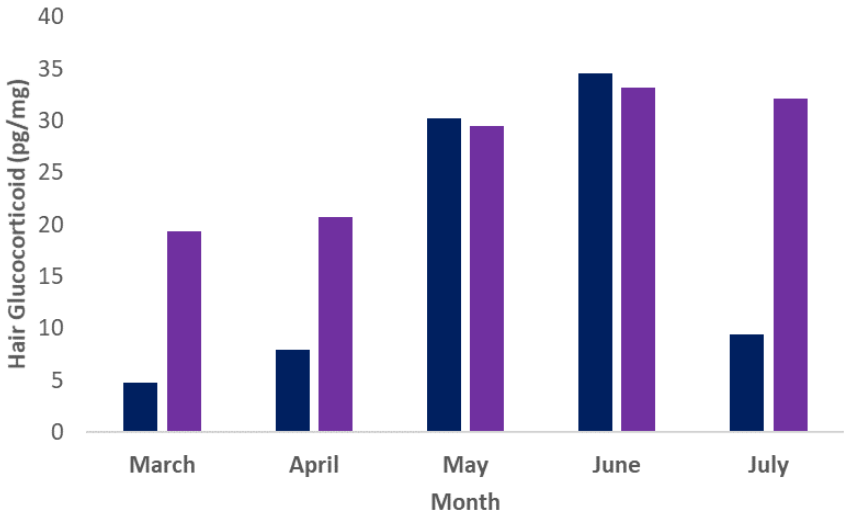
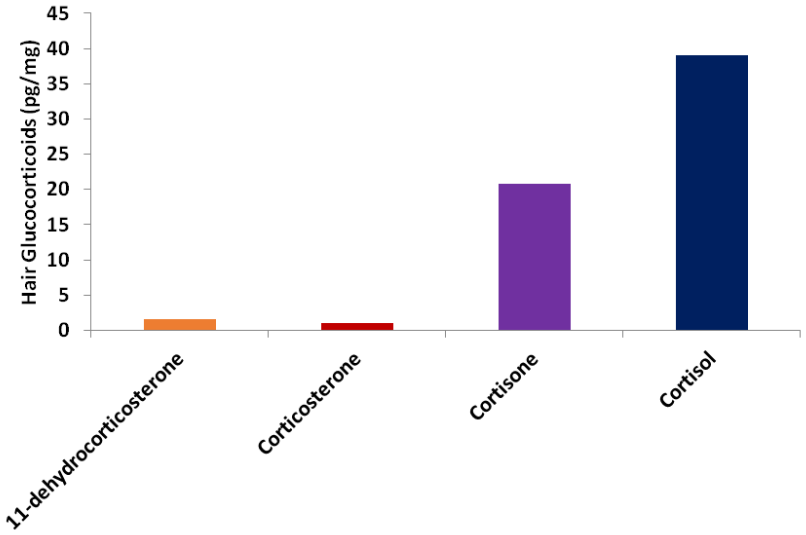


Figure 3-2: Glucocorticoid concentrations in 250 mg hair sample

Data are for concentrations of 11-dehydrocorticosterone (orange fill), corticosterone (red fill), cortisone (purple fill) and cortisol (blue fill) in hair sample from one healthy female individual.



3.2.3 Discussion

In this study of glucocorticoid measurement in human hair, we have detected cortisol and cortisone at similar concentrations to those published in healthy individuals. Corticosterone was not detectable in the standard hair sample but we have demonstrated it was present when a larger sample was analysed.

There can be wide variability in glucocorticoid measurements in hair. In a study of cortisol and cortisone measurements in 17 male and 27 female participants aged 2-90 years, cortisol and cortisone concentrations ranged from 5-91 pg/mg (mean 18 pg/mg) and 12-163 pg /mg (mean 70 pg/mg) respectively (Raul et al. 2004). Cortisone is generally reported at higher concentration than cortisol in hair (Stalder et al. 2013; Noppe et al. 2015). This is largely reflected in the 5 hair segments representing monthly exposure which is comparable with these results. The larger sample did not reflect this but is not representative of a one month hair segment and is therefore difficult to compare effectively.

Corticosterone was not detected using the initial method with the recommended amount of hair per sample (10-30 mg) (Manenschijn et al. 2011; Noppe et al. 2015). It had been anticipated that it may be difficult to detect corticosterone in hair considering the already low values for cortisol, the limits of detection of our analytical method and the relative expression of ABCC1 and ABCB1 in hair follicles. The limit of detection of corticosterone for this assay was 0.1 ng and assuming corticosterone might be present in hair at 10-fold lower concentration than cortisol, we estimated a sample of approximately 250 mg would be required. In combining the remaining hair sample,

we were able to analyse for glucocorticoids, but this was not representative of any relevant period of time as the full length of the lock of hair was used. Corticosterone was detectable in this large sample but at very low levels. The cortisol: corticosterone ratio was much lower than in plasma.

The reported relatively high expression of ABCC1 compared to ABCB1 in the hair follicle may play a role in the relatively low concentration of corticosterone in hair (Haslam et al. 2013). The mechanism of cortisol absorption in hair is not well understood although it is thought to come primarily from the blood supply along with some incorporation from sebum and sweat (Meyer & Novak 2012). Unlike plasma and saliva, there does not appear to be metabolism of glucocorticoids once incorporated into the hair shaft. This suggests hair glucocorticoid concentration is determined at the follicle and if ABCC1 is present, this would account for lower concentrations of corticosterone than in plasma.

Of note, 5α reductase (type 1 and 2) is present in hair follicles and has been targeted to prevent male pattern baldness (Batrinos 2014). Clearance of glucocorticoids via the 5α-reductase pathway is therefore likely to occur at this site however both cortisol and corticosterone would be metabolised through the same pathway.

A clear limitation in drawing conclusions from these data is that only one individual's hair has been analysed. This may not be representative and further analysis with multiple samples would be required to confirm our findings.

In practical terms, if our results are representative, in order to reliably detect corticosterone for monthly exposure in 1cm segments of hair, the volume required

would be over 250 mg. This represents a significant portion of hair and would not be feasible or acceptable as a clinical tool for measuring long term corticosterone exposure.

3.3 Salivary corticosterone

We hypothesised that salivary corticosterone would differ from cortisol in concentration due to ABC transporter expression. In this section, we aimed to measure corticosterone in saliva from healthy volunteers over a 14-hour period.

3.3.1 Methods

Saliva samples from a previous clinical study were obtained. This study investigated the association of 5α-reductase inhibition and metabolic dysfunction in males (Upreti et al. 2014). Six healthy male participants provided five salivary samples over the course of one day: on waking; 30 minutes after waking; at midday; at 6pm and at bedtime. Samples were taken from the placebo phase of this study.

3.3.1.1 Saliva collection

Participants were asked to collect each saliva sample using Salivette collection tubes (Sarstedt, Sarstedt, Germany). They were advised to avoid eating, drinking coffee/tea, brushing teeth and smoking cigarettes prior to sampling. On collection, saliva was subject to centrifugation (at 1912 g for 5 minutes at 4 °C) and frozen at -40 °C until analysis. Samples had already been through one freeze-thaw process prior to analysis for corticosterone.

3.3.1.2 Sample analysis

Saliva samples were analysed using immunoassay to measure corticosterone as described in section 2.4. Cortisol concentrations had been measured previously (Upreti 2013).

3.3.1.3 Statistical analysis

Data are mean ± SEM unless otherwise stated. Cortisol and corticosterone concentrations and corticosterone: cortisol ratio over the 14-hour period were compared using repeated measures analysis of variance (ANOVA) with post-hoc least square difference (LSD) testing. Data were analysed using SPSS version 23. $P<0.05$ was considered significant.

3.3.2 Results

Participant demographics are shown in Table 3-2.

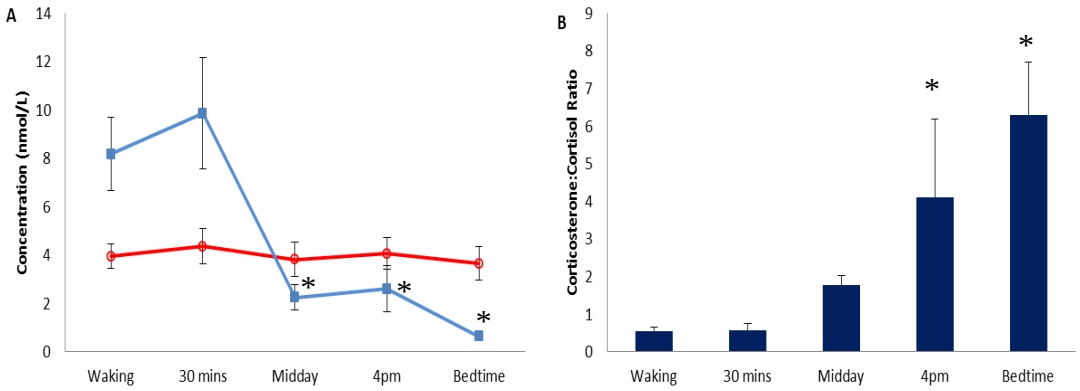
Table 3-2: Participant demographic data

	Mean ± standard deviation	**Range**
Age (years)	32.8±12.0	22.0-53.0
BMI (kg/m^2)	24.4±4.2	18.6-30.6
Blood Pressure:		
Systolic (mmHg)	126.2±11.5	114.0-143.0
Diastolic (mmHg)	75.5±9.3	65.0-89.0

There was diurnal variation of salivary cortisol with a peak at 30 minutes after waking and values thereafter significantly different to waking ($p<0.05$) (Figure 3-3). Corticosterone concentrations were not significantly different at any time point compared to waking values. The salivary corticosterone: cortisol ratio was high at baseline compared to usual circulating plasma ratio of approximately 0.05-0.1 and increased significantly over the course of the day ($p<0.05$).

Figure 3-3: Salivary cortisol and corticosterone by ELISA

*Data are mean ± SEM for A) salivary cortisol (blue line) and corticosterone (red line) and B) corticosterone: cortisol ratio at 5 different time points (n=6). Salivary cortisol but not corticosterone decreased from baseline (cortisol: p<0.05 v time, * significant on post hoc testing, corticosterone: p=0.38). The corticosterone: cortisol ratio increased from baseline (p<0.05 v time, * significant on post hoc testing).*



3.3.3 Discussion

In this small group of healthy individuals, we have shown salivary cortisol has a circadian rhythm over a 14-hour period but corticosterone is unchanged.

The salivary corticosterone concentrations in our study were higher than the few published studies which found early morning concentrations of 0.5 nmol/L (McVie et al. 1979) or indeed undetectable (Saracino et al. 2014). The reasons for this discrepancy are unclear but one reason may be the assays used in each study. It is possible that by measuring corticosterone by immunoassay, we have over-estimated the true value due to interference of other glucocorticoid metabolites. Cross reactivity in our assay was reported as 28.6% with deoxycorticosterone, 1.7% with progesterone and 0.046% with cortisol. Salivary deoxycorticosterone concentrations have been

measured at 0.23 nmol/L (Al-Dujaili et al. 2011) and progesterone in healthy males were reported at 0.26 nmol/L (Leisegang et al. 2014) suggesting they are unlikely to be causing significant interference with our results. The assay does not mention cross reactivity with 11-dehydrocorticosterone and the concentration of this in human saliva is as yet unknown.

The higher corticosterone concentrations may also be a reflection of the action of ABC transporters in salivary glands. A study of ABC expression in various tissues suggested that both ABCB1 and ABCC1 expression were relatively low in salivary glands compared to other tissues (Nishimura & Naito 2005) however a study of salivary glands alone found expression of both, with ABCB1 more highly expressed than ABCC1 in striated and excretory duct cells (Uematsu et al. 2003). With higher ABCB1 expression, there may be a relative excess of corticosterone due to export of cortisol.

The integrity of the samples is another possible confounder as they have all previously been used in other assays and had therefore been through the freeze- thaw process at least once. It may be expected that this would lead to degradation of the sample and under-estimation of the concentration however.

The lack of diurnal variation of corticosterone was unexpected. We are unaware of any previously published data of salivary corticosterone throughout a wake/sleep cycle so we have no comparisons for this finding. This raises the question of whether salivary corticosterone truly reflects plasma concentrations or whether plasma corticosterone has a less defined circadian rhythm.

With limited published data on salivary corticosterone, it was difficult to be certain whether our results were reproducible. Further investigation of these findings with paired plasma and saliva samples and LC-MS/MS analysis was therefore performed to improve our understanding of corticosterone physiology.

3.4 Paired plasma and saliva cortisol and corticosterone measurements in healthy individuals

3.4.1 Introduction

The results from section 3.3 suggest that salivary corticosterone does not have a diurnal rhythm unlike cortisol. We hypothesised that salivary corticosterone does not correlate with plasma corticosterone and that only plasma corticosterone has a circadian rhythm.

We aimed to investigate the relationship between salivary and plasma corticosterone in healthy individuals by measuring paired plasma and saliva samples over a 14-hour period. To improve detection and specificity, we optimised a new LC-MS/MS method to measure both cortisol and corticosterone.

3.4.2 Methods

3.4.2.1 Ethical and research governance approvals

This study was approved by the ACCORD Medical Research Ethics Committee (AMREC) (16-HV-029) and NHS Lothian Research and Development committee (2017/0004). Research support approvals were secured with the Wellcome Trust Clinical Research Facility (WTCRF) and NHS Lothian laboratories at the Royal Infirmary of Edinburgh (RIE).

3.4.2.2 Study design

A healthy volunteer study was performed to measure glucocorticoid concentrations in plasma and saliva over a 14-hour period (from 8am to 10pm). Participants attended for one study day and paired plasma and saliva samples were taken at regular intervals.

3.4.2.3 Participants

10 participants were recruited via advertisement on social noticeboards and from contacting individuals who had previously taken part in clinical studies. Subjects were then invited for a screening visit to discuss the study in detail and to obtain written informed consent. Eligibility was assessed through acquisition of relevant medical history, clinical examination and baseline blood tests.

3.4.2.3.1 Inclusion criteria

- Aged 18-60 years
- Male or female

3.4.2.3.2 Exclusion criteria

- Current acute or chronic medical condition
- Exogenous glucocorticoid use by any route in last 3 months
- Any regular medication use
- Abnormal screening bloods (full blood count (FBC), urea and electrolytes (U+E), thyroid function tests (TFTs), liver function tests (LFTs), random glucose)
- Alcohol intake >14 units/week

3.4.2.4 Measurements

Height, weight (clothed), BMI, hip and waist circumference were measured as per standard operating procedure. A self-standing height measurement was used to measure height to the vertex of the head with their shoes off and back of heels and head against the measuring board (to one decimal place). Clothed weight was

measured before the first visit on calibrated electronic scales (SECA 704, SECA Electronic Scales, Hamburg, Germany). Waist circumference was measured mid-way between the costal margin and the iliac crest with the participant standing. Hip circumference was measured around the maximum circumference of the buttocks in the standing position (World Health Organisation 2008). Percentage body fat and lean body mass were measured using the mean of three recordings by bioelectrical impedance on the morning of each visit (OMRON BF302, OMRON Healthcare (UK) Ltd, Henfield, UK). Blood pressure and pulse (OMRON 705IT BP monitor, OMRON Healthcare (UK) Ltd, Henfield, UK) were recorded with the participant sitting.

3.4.2.5 Sample size calculation

Previous studies have shown correlation between plasma and salivary cortisol concentration with correlation coefficients of between 0.61-0.94 (Vining et al. 1983; Thomson et al. 2007). Assuming a moderate correlation between plasma and salivary corticosterone of 0.5, 10 subjects provides >0.9 power to be able to reject the null hypothesis that there was no correlation between plasma and saliva corticosterone concentrations. The Type 1 error probability associated with the test of this null hypothesis was 0.05.

3.4.2.6 Clinical protocol

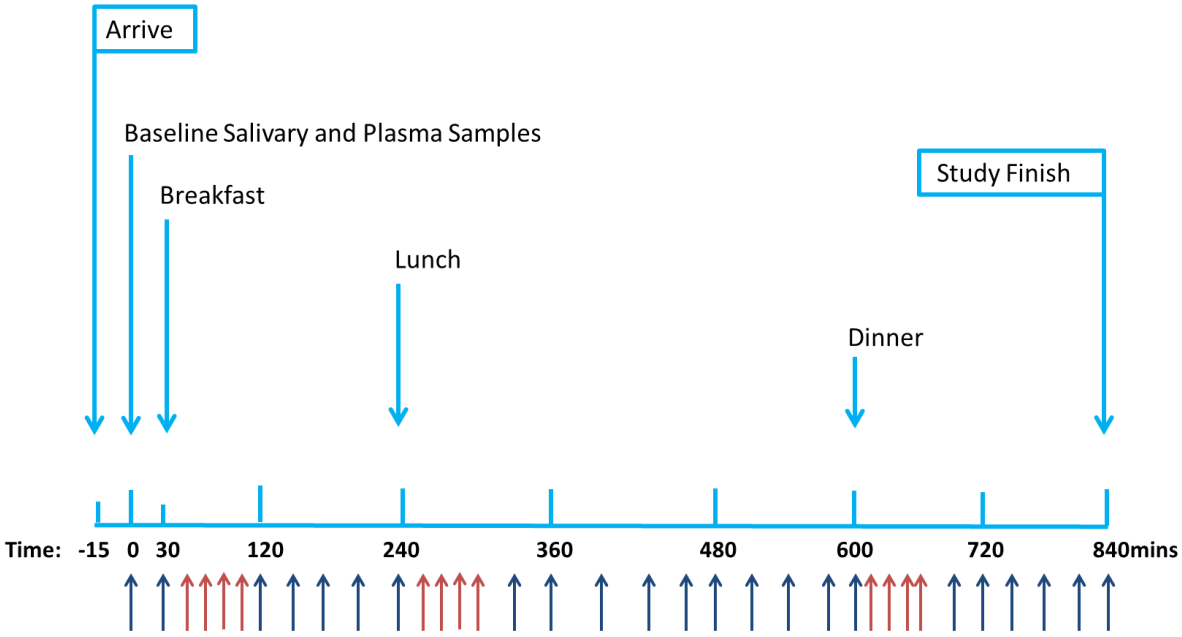
3.4.2.6.1 Study visits

Participants attended the WTCRF at 7.45am following an overnight fast. On arrival, participants had height, weight and blood pressure measured. Lean body mass and fat mass using bioelectrical impedance analysis was measured as in section 3.4.2.4. A 20

gauge (20G) (Braun, Sheffield, UK) cannula was inserted in the antecubital fossa under aseptic technique.

Figure 3-4: Study protocol

Paired blood and saliva sampling was carried out every 30 minutes (blue arrows) with more frequent sampling every 15 minutes after meals for 1 hour (red arrows).



Study visits commenced at 8am (Time (t) = 0 mins) when the first paired plasma and saliva samples were taken. Sampling was every 30 minutes thereafter. Meals were given at 8.30am (t+30 mins), 12 noon (t+240 mins) and 6pm (t+600 mins) of set nutritional content (Figure 3-4). Paired blood and saliva sampling was performed every 15 minutes after meals for 1 hour before reverting to 30 minute sampling again. Meals consisted of 55% carbohydrate, 15% protein and 30% fat. Calorie content was 350 kcal, 500 kcal and 700 kcal for breakfast, lunch and dinner respectively. After the final sample at 10pm, the cannula was removed and subjects were allowed home.

3.4.2.6.2 Sample collection and processing

Plasma samples were obtained in lithium heparin (2.9 mL) tubes (Monovette®, Sarstedt, Numbrecht, Germany) and immediately placed on ice. Samples were gently mixed by inverting several times. Blood samples were subject to centrifugation immediately after sampling (2000 g for 10 minutes at 4°C) and the plasma was removed and stored at -80°C prior to analysis.

Saliva samples were obtained using the Salivette® saliva collection device (Sarstedt, Numbrecht, Germany). Subjects chewed on the Salivette® for 1 minute before placing it back in the collection tube. This was centrifuged immediately (2200 g for 10 minutes at 20 °C) after sampling, separated and stored at -80°C for analysis.

3.4.2.7 Sample analysis

3.4.2.7.1 Preparation of standard curves

Standard curves were prepared for plasma and saliva analytes in Table 3-3 and Table 3-4 as described in section 2.3.2.

Table 3-3: Standard curve analytes and internal standards for plasma

Analyte	Standard Curve Range (ng)	Internal Standard	Internal Standard amount (ng)
Cortisol	0.01-200	D4-cortisol	50
Cortisone	0.01-200	D4-cortisol	50
Corticosterone	0.001-20	Epi-corticosterone	10
11-dehydrocorticosterone	0.001-20	Epi-corticosterone	10

Table 3-4: Standard curve analytes and internal standards for saliva

Analyte	Standard Curve Range (ng)	Internal Standard	Internal Standard amount (ng)
Cortisol	0.0005-10	D4-cortisol	0.5
Cortisone	0.0005-10	D4-cortisol	0.5
Corticosterone	0.0005-10	Epi-corticosterone	0.5
11-dehydrocorticosterone	0.0005-10	Epi-corticosterone	0.5

3.4.2.7.2 Extraction of samples

Plasma and saliva samples were extracted as described in sections 2.3.3 and 2.3.4 respectively.

3.4.2.7.3 LC-MS/MS analysis

Both plasma and salivary samples were analysed as described in section 2.3.6.2.

3.4.2.8 Statistical analysis

All data presented are mean ± SEM unless otherwise stated. Data were analysed using SPSS (version 23, IBM SPSS Statistics, Portsmouth, UK). Plasma and salivary measurements were compared independently with time using repeated measures analysis of variance (ANOVA) and least squares difference (LSD) post-hoc testing. Samples below the limit of quantitation were assigned the value of that limit. Plasma and saliva measurements were analysed for correlation using Pearson's correlation if normally distributed and Spearman's Rank correlation if not normally distributed. Correlation plots presented show mean data for each time point. $P<0.05$ was considered significant.

3.4.3 Results

3.4.3.1 Characteristics of study participants

Anthropometric data from the ten healthy volunteers (n=5 female) are presented in Table 3-5. During the study visits, two participants (one female) fainted during insertion of the peripheral cannula. Although the study visit continued after they recovered, glucocorticoid measurements were up to 5-fold higher than expected and they were therefore excluded from analysis as outliers.

Table 3-5: Participant demographic data

	Mean ± standard deviation	**Range**
Age (years)	27.2±6.3	20.0-40.0
BMI (kg/m^2)	25.8±3.9	22.1-32.7
Bioimpedance:		
% Fat	22.1±9.0	6.6-35.8
Fat Mass (kg)	17.3±7.9	4.5-30.4
Blood Pressure:		
Systolic	123.0±11.6	96.0-138.0
Diastolic	75.7±5.0	70.0-85.0

3.4.3.2 Plasma and saliva glucocorticoids

3.4.3.2.1 Corticosterone

Plasma corticosterone peaked in the morning and fell during the day with a second peak mid-afternoon (Figure 3-5). There was no increase in corticosterone following meals. Salivary corticosterone was very low and 42% of samples were at or below the limit of detection of the assay. Detectable concentrations of corticosterone did not

show any diurnal variation (p=0.44). Subject 10 had no salivary corticosterone values below the limit of detection and diurnal variation was not apparent compared with plasma concentration (Figure 3-5 B). Salivary and plasma concentrations did not correlate when compared with Spearman's rank correlation.

3.4.3.2.2 11-dehydrocorticosterone

There was diurnal variation of 11-dehydrocorticosterone in plasma with a second peak in mid-afternoon with no increase following meals (Figure 3-6). Salivary 11-dehydrocorticosterone also followed a diurnal rhythm and was present in higher concentrations than corticosterone ($p<0.05$ vs time, $p=0.02$ vs corticosterone). Only 9.3% of samples were below the limit of quantitation of the assay. Salivary 11-dehydrocorticosterone correlated significantly with plasma concentrations of both 11-dehydrocorticosterone and corticosterone ($r=0.6720$, $p<0.0001$ and $r=0.7154$, $p<0.0001$).

Figure 3-5: Plasma and salivary corticosterone

Data are mean ± SEM for A) plasma corticosterone (red unbroken line) on left axis and salivary corticosterone (red dashed line) on right axis (n=8). Comparison was tested using repeated measures ANOVA. Plasma corticosterone changed with time (p<0.0001 vs time) but salivary corticosterone was unchanged (p=0.44 vs time). Plasma (red unbroken line) on left axis and salivary (red dashed line) corticosterone on right axis for Subject 10 is shown in B. Correlation of salivary and plasma corticosterone is shown in C (n=8). Correlation was tested with Spearman's rank correlation. There was no correlation of plasma and salivary corticosterone (r=-0.07, p=0.6788).

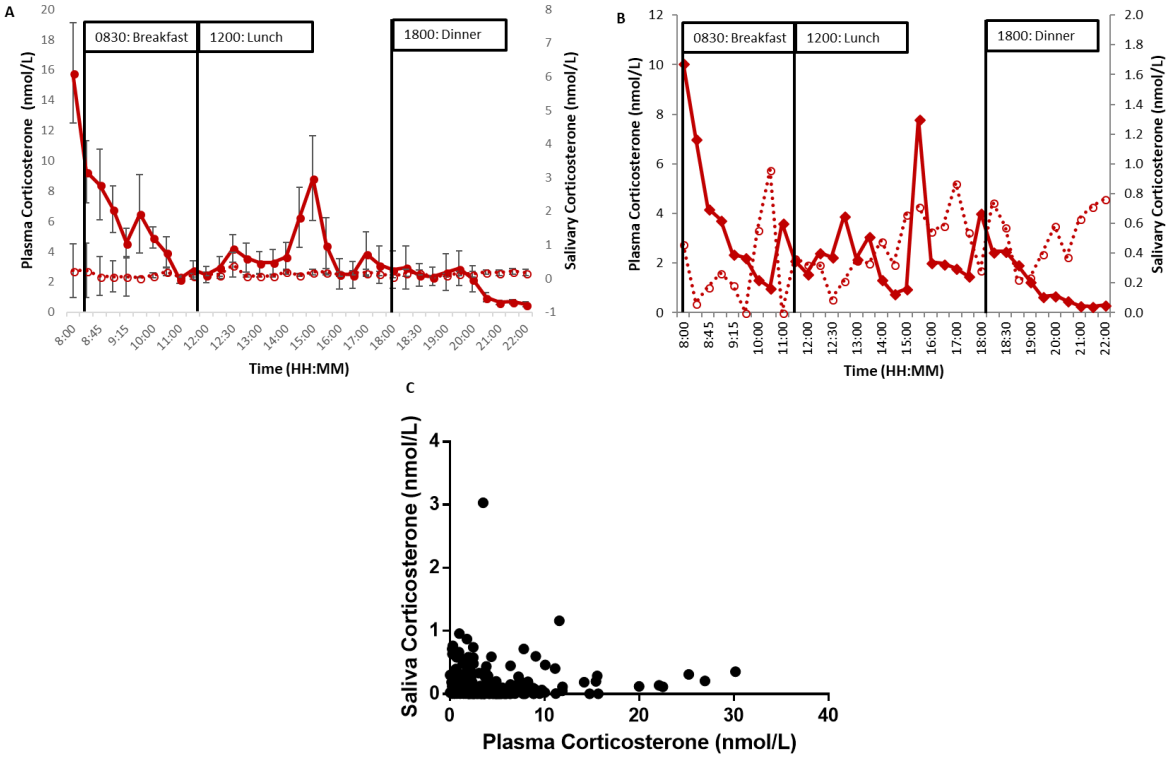
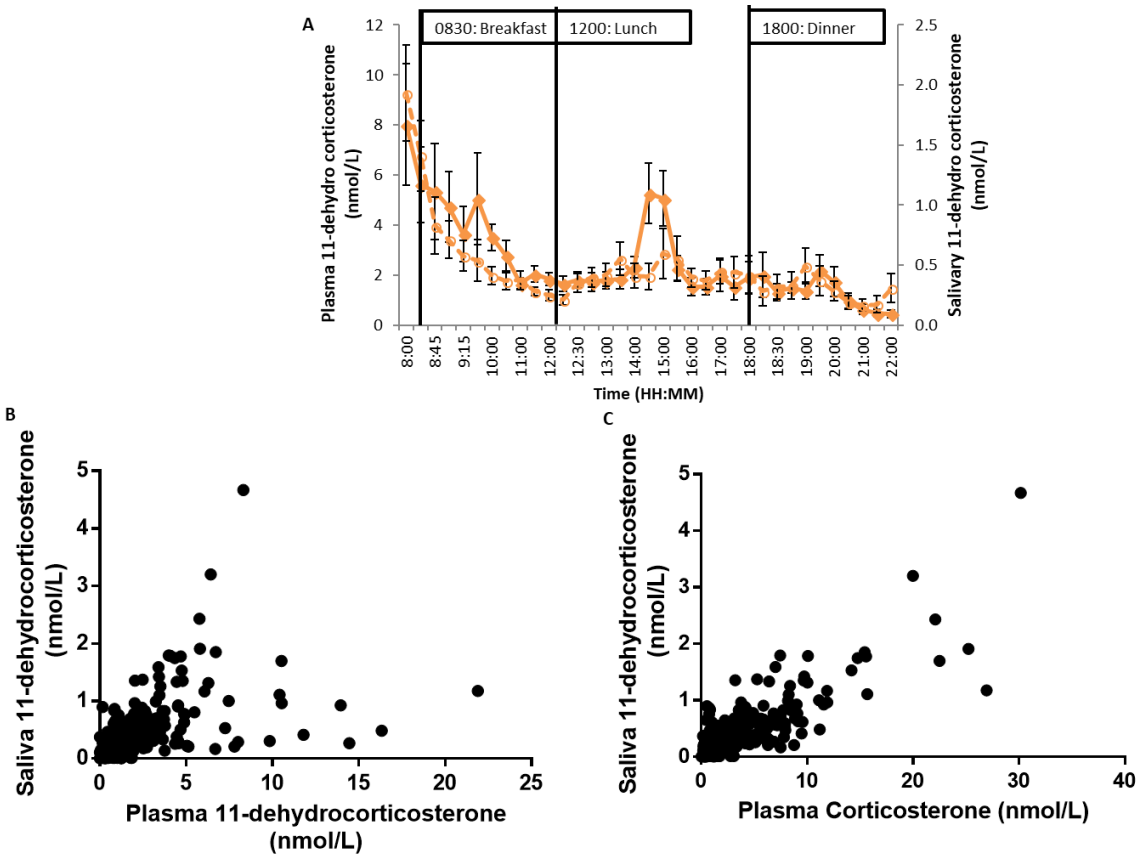


Figure 3-6: Plasma and salivary 11-dehydrocorticosterone

Data are mean ± SEM for A) plasma 11-dehydrocorticosterone (orange unbroken line) on left axis and salivary 11-dehydrocorticosterone (orange dashed line) on right axis (n=8). Comparison was tested using repeated measures ANOVA. Both plasma and salivary 11-dehydrocorticosterone changed significantly with time (both p<0.05 vs time). Correlation of salivary and plasma 11-dehydrocorticosterone is shown in B (n=8). Correlation was tested with Spearman's rank correlation. There was significant correlation of plasma and salivary 11-dehydrocorticosterone (r=0.6720, p<0.0001). Correlation of salivary 11-dehydrocorticosterone and plasma corticosterone is shown in C (n=8). There was significant correlation of salivary 11-dehydrocorticosterone and plasma corticosterone (r=0.7154, p<0.0001).

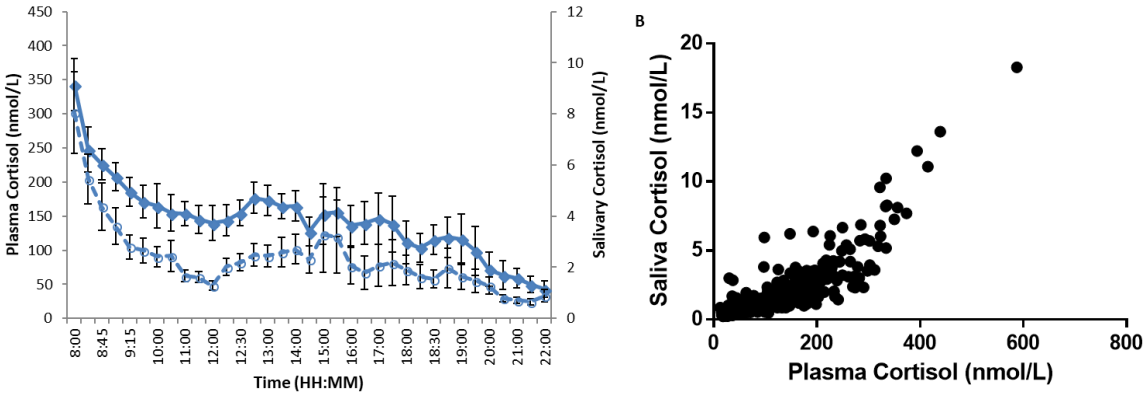


3.4.3.2.3 Cortisol

Both plasma and salivary cortisol followed a classic circadian rhythm (Figure 3-7). There was a small peak after lunch and dinner. Correlation of plasma and salivary concentrations was significant (r=0.8812, p<0.0001)

Figure 3-7: Plasma and salivary cortisol

Data are mean ± SEM for A) plasma cortisol (blue unbroken line) on left axis and salivary cortisol (blue dashed line) on right axis (n=8). Comparison was tested using repeated measures ANOVA. Both plasma and salivary cortisol changed significantly with time (both p<0.05 vs time). Correlation of salivary and plasma cortisol is shown in B (n=8). Correlation was tested with Spearman's rank correlation. There was significant correlation of plasma and salivary cortisol (r=0.8812, p<0.0001).

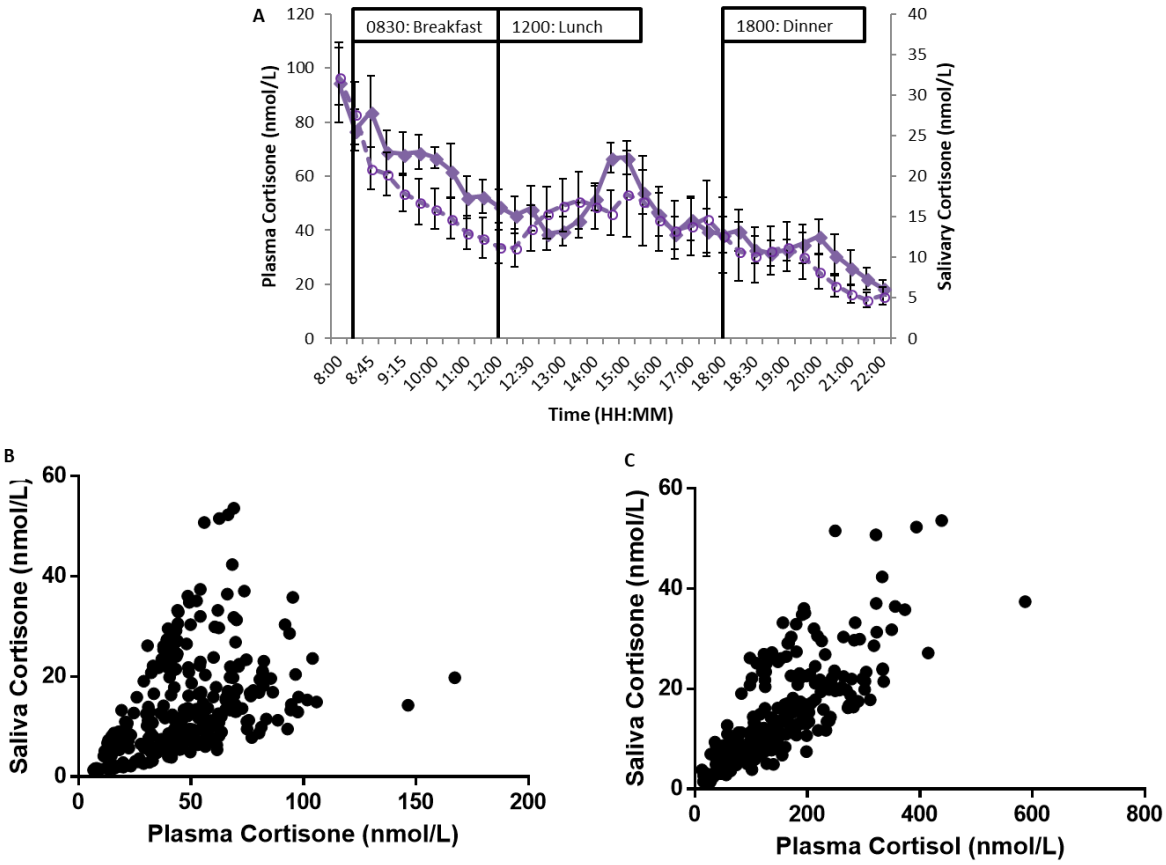


3.4.3.2.4 Cortisone

There was diurnal variation of cortisone in both plasma and saliva (Figure 3-8). Salivary concentrations were greater than measured cortisol concentrations (p=0.001 vs cortisol). There was good correlation between plasma and salivary concentrations (r=0.8778, p<0.0001) and salivary cortisone correlated well with plasma cortisol (r=0.9650, p<0.0001).

Figure 3-8: Plasma and salivary cortisone

Data are mean ± SEM for A) plasma cortisone (purple unbroken line) on left axis and salivary cortisone (purple dashed line) on right axis (n=8). Comparison was tested using repeated measures ANOVA. Both plasma and salivary cortisone changed significantly with time (both p<0.001 vs time). Correlation of salivary and plasma cortisone is shown in B (n=8). Correlation was tested with Pearson's correlation. There was significant correlation of plasma and salivary cortisone (r=0.8778, p<0.0001). Correlation of salivary cortisone and plasma cortisol is shown in C (n=8). There was significant correlation of plasma cortisol and salivary cortisone (r=0.9650, p<0.0001).

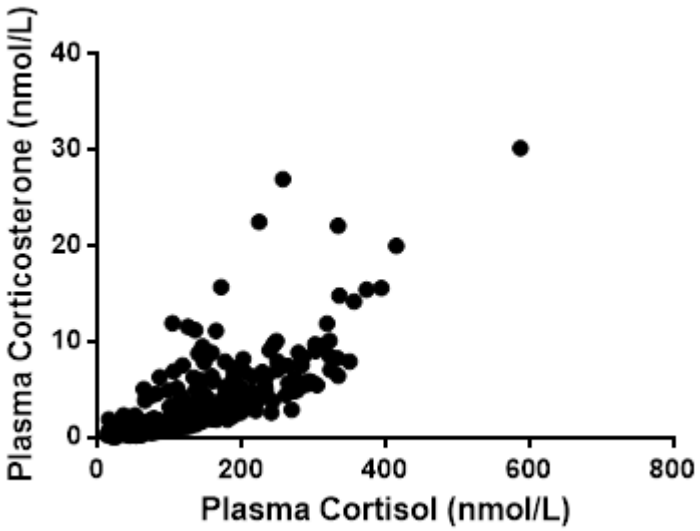


3.4.3.3 Relationship between cortisol and corticosterone in plasma and saliva

The plasma concentrations of cortisol and corticosterone followed a similar diurnal pattern and there was significant correlation (Figure 3-9).

Figure 3-9: Correlation of plasma cortisol and corticosterone

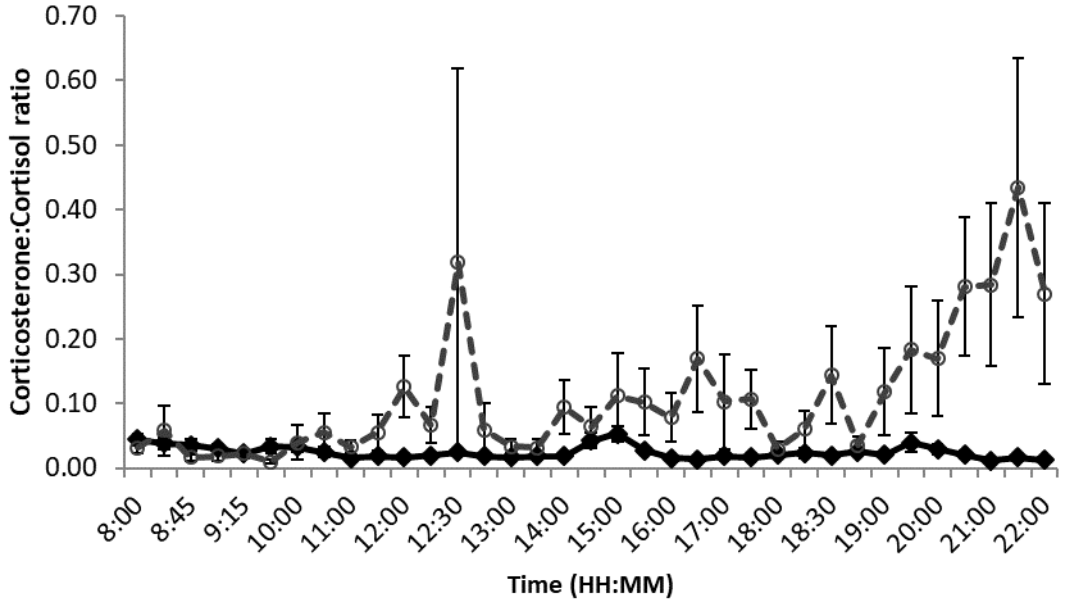
Correlation of plasma cortisol and corticosterone is shown (n=8). Correlation was tested with Spearman's rank correlation. There was significant correlation of plasma cortisol and corticosterone (r=0.8182, p<0.0001).



The corticosterone: cortisol ratio in saliva and plasma was similar at baseline and despite an apparent rise over the study period in saliva, there was no significant difference between the ratio in the different biological samples (Figure 3-10).

Figure 3-10: Corticosterone: cortisol ratio in plasma and saliva

Data are mean ± SEM for corticosterone: cortisol ratio in plasma (black unbroken line) and saliva (grey dashed line) (n=8). Comparison was tested using repeated measures ANOVA. There was no significant difference between salivary and plasma corticosterone: cortisol ratio although there was a trend for the salivary ratio to increase with time (p=0.07).



3.4.4 Discussion

This study of glucocorticoid concentrations in paired plasma and saliva of healthy volunteers has shown that corticosterone does have a diurnal variation in plasma but not in saliva, where salivary corticosterone is low in concentration. In contrast, both plasma and salivary concentrations of 11-dehydrocorticosterone, cortisol and cortisone have a diurnal rhythm and correlate significantly with each other.

The concentration of corticosterone in saliva is much lower than our results in section 3.2 but corroborate better with previously published data (McVie et al. 1979).

Interestingly, McVie et al used radioimmunoassay in their analysis and the main drawback of immunoassay use is over-estimation of steroid content due to cross-reactivity with other substances (Keevil 2013). While this is likely to be the cause of the elevated values in the previous section, McVie et al appear to have a more specific assay which correlates with our current data using LC-MS/MS analysis.

We chose to measure salivary corticosterone using LC-MS/MS which allows a more sensitive and specific method of detection and simultaneous measurement of several glucocorticoids (Turpeinen & Hämäläinen 2013). Our method was optimised to allow the analysis of 400 μL of saliva with a limit of quantitation (LOQ) of 0.005 ng. Other methods have been published using only 100 μL of saliva with LOQ < 0.005 ng (Gao et al. 2015). In addition to testing the validity of their method, they also measured steroid content of 16 saliva samples from a previous clinical study. Corticosterone measurements ranged from 0-1.5 nmol/L with a mean±SD of 0.184±0.39 nmol/L. This range is similar to the results in this study. The sensitivity of our assay was variable and our LOQ ranged between 0.005-0.025 ng which meant a number of samples were at the very limits of detection.

With 43% of the salivary corticosterone measurements below the limit of detection for our assay, this represents a significant limitation of this study. We were unable to detect any diurnal variation but this may simply be lost due to the lack of sensitivity. There was no significant correlation between salivary and plasma corticosterone but it is difficult to conclude there is no correlation at all without being able to quantify the true salivary concentrations. In one subject who had detectable salivary corticosterone

throughout, there was no clear correlation with plasma concentrations. Although salivary corticosterone was low in concentration, the corticosterone: cortisol ratio was similar at baseline for plasma and saliva.

While the salivary corticosterone concentrations may simply be proportional to the circulating plasma levels, there may be further metabolism of corticosterone in saliva which would account for the lack of observed diurnal rhythm. There is significant activity of 11β-HSD2 in the parotid gland (Smith et al. 1996) and as a result cortisone is widely reported to be present in higher concentrations than cortisol in saliva (Perogamvros et al. 2009; Mezzullo et al. 2016). Corticosterone is also subject to inactivation by 11β-HSD2 and we can confirm this in our study with 11-dehydrocorticosterone salivary concentrations significantly higher than corticosterone. It is possible that the conversion is sufficient to result in such low substrate levels that any diurnal variation is lost.

The lack of correlation of plasma and salivary corticosterone suggests that without a more sensitive assay, saliva is not a useful marker of plasma corticosterone. Similar to cortisone and cortisol however, our data show 11-dehydrocorticosterone is an effective marker of plasma corticosterone. In fact, salivary cortisone rather than cortisol has been suggested as a better measure to reflect plasma cortisol and our data reflects this with better correlation of plasma cortisol with salivary cortisone (Perogamvros, Keevil, et al. 2010). The same appears to be true for corticosterone and 11-dehydrocorticosterone.

In contrast, plasma corticosterone had a circadian rhythm similar to cortisol and mirrored the limited published data available for repeated daily measurements. Martin et al measured plasma corticosterone at 6 time points over a 24 hour period using a fluorometric method and concentrations ranged from 17.3 nmol/L at 9am to 5 nmol/L at midnight (Martin & Martin 1968). Our data adds to the published literature suggesting plasma corticosterone too has a circadian rhythm. The positive correlation of plasma cortisol and corticosterone suggest they are under similar regulation within the circulation.

There is evidence that eating influences cortisol concentrations with peaks seen after meals (Quigley & Yen 1979; Follenius et al. 1982). More recent data suggests this is mediated by a combination of adrenal secretion and extra-adrenal regeneration depending on macronutrient meal content (Stimson et al. 2014). Protein and fat lead to a cortisol rise largely mediated by adrenal secretion whereas the contribution of adrenal and extra-adrenal regeneration was roughly equal after carbohydrate. There is no published evidence of the effect meals have on circulating corticosterone in humans. Our data support the literature with small peaks observed in plasma cortisol after lunch and dinner. The response of plasma corticosterone is less clear, with a small discernible peak after lunch but little change after dinner. There does appear to be a peak in the mid-afternoon around 3pm which is also seen in plasma 11-dehydrocorticosterone and to a lesser extent in plasma cortisone levels. The reason for this delayed peak in plasma levels is unclear. The meals provided were of identical nutrient content with a set ratio of carbohydrate, protein and fat. A delayed secretion of adrenal corticosterone seems unlikely since cortisol would follow the same pattern.

A delay in regeneration of corticosterone via 11β-HSD1 is possible and this would require further investigation with *in vivo* tracer studies.

In conclusion, we have shown corticosterone has a circadian rhythm in plasma similar to cortisol. In saliva, corticosterone concentrations were low and appeared to lack a diurnal rhythm. Until a more sensitive assay is available, salivary 11-dehydrocorticosterone would be a suitable alternative as a non-invasive measure of plasma corticosterone. In scalp hair, corticosterone was only detectable in a very large sample and based on this preliminary work, clinical use is likely to be limited.

Chapter 4. The role of ABCC1 in glucocorticoid transport *in vivo*

4.1 Introduction

Intracellular glucocorticoid concentration is determined in a tissue specific manner by the expression of ABC transporters (Ueda et al. 1992; Karssen et al. 2001; Nixon et al. 2016). ABCB1 is most highly expressed in brain, adrenals and small intestine and exports cortisol but not corticosterone (Karssen et al. 2001; Karssen et al. 2004; Nishimura & Naito 2005; Begley 2004). ABCC1 is more widely expressed although primarily in adipose tissue, skeletal muscle and thyroid and conversely exports corticosterone but not cortisol (Nixon et al. 2016; Nishimura & Naito 2005; Webster & Carlstedt-Duke 2002).

Although circulating plasma corticosterone concentrations are relatively low compared to cortisol, the tissue-specific differential expression of these transporters allows corticosterone to have a more or less significant role in certain tissues. In brain, for example, the predominant expression of ABCB1 over ABCC1 at the blood brain barrier would suggest a more significant role for corticosterone in central hypothalamic negative feedback of the HPA axis (Karssen et al. 2001). This is supported by evidence of increased concentration of corticosterone in CSF and post-mortem brain specimens compared to circulating levels (Raubenheimer et al. 2006). Negative feedback is mediated at the level of the pituitary, hypothalamus and higher limbic structures and only the pituitary is out with the blood brain barrier. This would suggest corticosterone plays more of a key role in these central areas of HPA negative feedback rather than in the pituitary itself.

Peripherally, selective ABCC1 rather than ABCB1 expression in skeletal muscle and adipose tissue may minimise the effects of corticosterone. Pharmacological inhibition and genetic knock out of ABCC1 increased corticosterone activation of corticosteroid receptors *in vitro* and in mice (Nixon et al. 2016). In a study of patients with Addison's disease, corticosterone and cortisol infused to achieve similar circulating concentrations resulted in comparable suppression of ACTH but higher expression of the acutely responsive glucocorticoid-responsive transcript *PER1* in adipose tissue in the cortisol phase (Nixon et al. 2016). This suggests that while cortisol and corticosterone induce equivalent suppression of the HPA axis, activation of intracellular receptors in adipose tissue is greater with cortisol. This may be due to export of corticosterone from adipose tissue cells by ABCC1 leading to reduced activation of corticosteroid receptors.

The export of corticosterone by ABCC1 is significant when we consider those who require glucocorticoid replacement therapy with hydrocortisone, the most widely used treatment (Forss et al. 2012). Many are maintained on supra-physiological doses due, in part, to the complexity of accurately mimicking the natural circadian release of glucocorticoids (Peacey et al. 1997). These patients can develop Cushingoid features and have significant morbidity and mortality secondary to cardiovascular disease (Falhammar et al. 2014).

If the actions of ABCB1 and ABCC1 in *in vitro* and murine models are similar in humans, this mechanism may be exploited to potentially improve outcomes in patients taking glucocorticoid replacement. ABCC1 expression in adipose tissue and skeletal

muscle is significant given the role these tissues play in development of metabolic syndrome (Morton 2010). Using corticosterone as an alternative replacement therapy may allow adequate glucocorticoid cover with less accumulation within adipose tissue and skeletal muscle and therefore potentially less metabolic side effects.

The effects of ABCC1 inhibition in humans have not yet been tested directly. The clinical study presented in this chapter aimed to determine whether inhibition of ABCC1 would affect the central negative feedback of the HPA axis and occupancy of the corticosteroid receptors in the peripheral tissues, adipose and skeletal muscle. We hypothesised that ABCC1 inhibition would not affect the HPA axis but would increase occupancy (and activation) of corticosteroid receptors by corticosterone, but not cortisol, in adipose and skeletal muscle.

In order to test this hypothesis, central negative feedback of the HPA axis had to be demonstrated and in addition, occupancy of GR and MR in adipose tissue and skeletal muscle measured. The GR and MR antagonists mifepristone (RU486) and potassium canrenoate were given to activate the HPA axis centrally and displace bound glucocorticoids peripherally. This technique has been used previously to measure occupancy of MR by cortisol in the myocardium (Iqbal et al. 2014). ABCC1 inhibition was achieved using the uricosuric agent probenecid which inhibits renal excretion of organic anions and reduces tubular reabsorption of urate. Historically used as treatment for chronic gout in humans, it has also been used as a synergistic treatment with antimicrobials (Cunningham et al. 1981; Robbins et al. 2012). Probenecid has been

used experimentally to inhibit ABCC1 both *in vitro* and *in vivo* (Webster & Carlstedt-Duke 2002; Nixon et al. 2016).

4.2 Methods

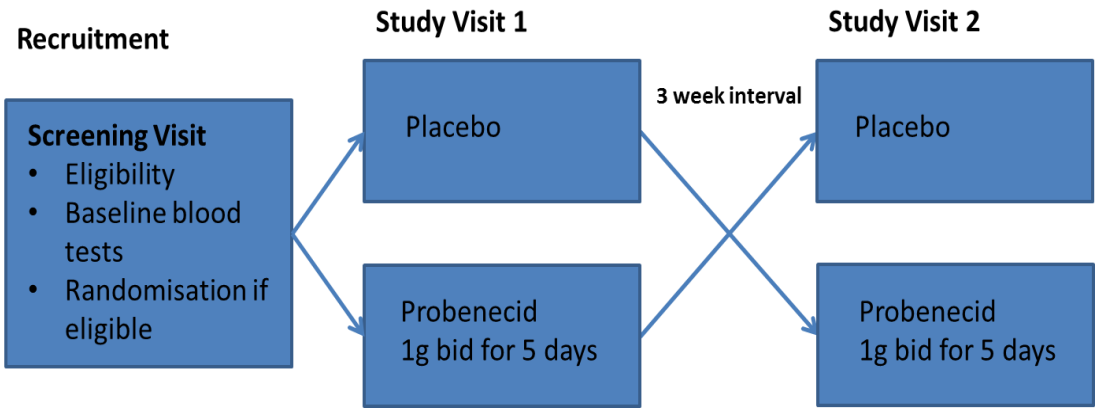
4.2.1 Ethical and research governance approvals

This study was approved by the Scotland A Research Ethics Committee (15/SS/0034) and by NHS Lothian Research and Development (2015/0200). Research support approvals were secured with the Royal Infirmary of Edinburgh Clinical Research Facility (RIECRF), NHS Lothian laboratories and radiopharmacy at the Royal Infirmary of Edinburgh (RIE).

4.2.2 Study design

A double blind randomised crossover study was performed in healthy men comparing the displacement of cortisol and corticosterone by corticosteroid receptor antagonists with simultaneous arterio-venous sampling from adipose tissue and skeletal muscle in the presence and absence of the ABCC1 inhibitor, probenecid. Participants attended on two occasions and were given either placebo or probenecid for 5 days prior to each visit (Figure 4-1). Displacement of cortisol and corticosterone was achieved using the MR antagonist potassium canrenoate and GR antagonist mifepristone (RU486). 1,2-$[^2H]_2$-cortisone (D2-cortisone) was infused to allow *in vivo* kinetic calculations of whole body and tissue glucocorticoid appearance rates. Blood flow was measured in adipose tissue and skeletal muscle by ^{133}Xenon gas washout and venous occlusion plethysmography respectively. Adipose tissue biopsies were taken before and after displacement to compare mRNA expression of known glucocorticoid-regulated genes.

Figure 4-1: Study design



4.2.3 Participants

Participants (n=14) were recruited through advertisement in a local newspaper and with posters. Subjects initially attended the RIECRF for a screening visit to discuss the study in detail and to obtain written informed consent. Eligibility was assessed through acquisition of relevant medical history, clinical examination and baseline blood tests.

4.2.3.1 Inclusion criteria

- Males aged 18-60 years old
- BMI 20-30 kg/m^2

4.2.3.2 Exclusion criteria

- Current acute or chronic medical condition (including history of kidney stones)
- Exogenous glucocorticoid use by any route in the past 3 months

- Abnormal screening bloods (full blood count (FBC), urea and electrolytes (U+E), thyroid function tests (TFTs), liver function tests (LFTs), random glucose)
- Any regular medication
- Alcohol intake >21units/week
- Venous anatomy that precludes adipose vein cannulation

4.2.4 Sample size calculation

We planned a study of a continuous response variable from within-subject pairs of measurements. Prior data from a myocardial release study was used to power this current protocol, in which cortisol release from the myocardium was measured after MR blockade (Iqbal et al. 2014). The mean change in cortisol concentration from steady state to maximal release (which occurred 25 minutes following canrenoate) was normally distributed at 55.3 with a standard deviation of 69.1. Based on this, 14 pairs of subjects were required in order to reject the null hypothesis that this response difference was zero with probability (power) 0.8. The Type 1 error probability associated with this test of this null hypothesis was 0.05.

4.2.5 Clinical protocol

4.2.5.1 Study visits

Participants attended on two occasions at least three weeks apart to allow washout between visits. They took either placebo or probenecid (Arena Pharmaceuticals Ltd, Buckingham, UK) 1g twice daily for 5 days prior to each study visit. Capsules were manufactured by Tayside Pharmaceuticals in sterile conditions. Participants were

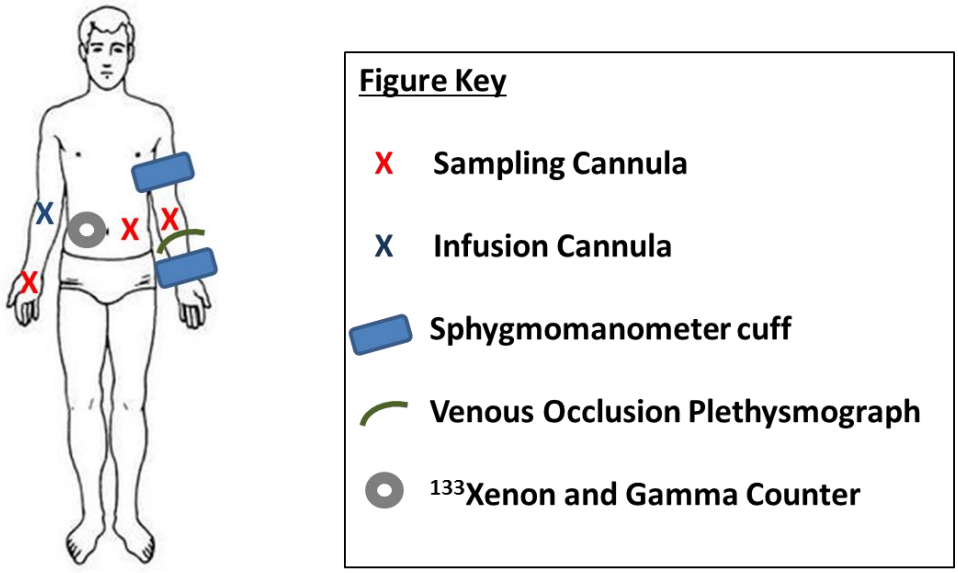
given written instructions to take 2 x 500mg capsules twice daily (8am and 8pm) for 5 days before each study visit, with the last 2 capsules taken on the morning of the visit. Participants were given placebo and probenecid in random order. Randomisation was undertaken by Tayside Pharmaceuticals and kept securely in a sealed envelope until all measurements were complete.

Participants attended the RIECRF at 8am having fasted from 10pm the night before. On arrival, measurements were taken of clothed height, weight, waist and hip circumference. Blood pressure, lean body mass and fat mass were also measured as described in section 3.4.2.4. A standardised breakfast was supplied consisting of 55% carbohydrate, 30% fat and 15% protein totalling 350 kcal.

The participant was cannulated using an aseptic technique at three sites for blood sampling and one further site for infusions (Figure 4-2). A retrograde 20G intravenous cannula (Braun, Sheffield, UK) was inserted in the deep branch of the medial cubital vein in the antecubital fossa of one arm for forearm skeletal muscle sampling. An inflatable cuff was placed at the wrist and inflated to 200 mmHg for 2 minutes prior to sampling to minimise contamination of blood from the hand. A further retrograde cannula was inserted in the dorsum of the hand on the contralateral side. The hand was placed in a hot box (manufactured in house) heated to 60°C for 5 minutes prior to sampling in order to obtain arterialised samples. This technique has been shown to mimic arterial blood and has been used in previous clinical studies to avoid the need for invasive arterial cannulation (Roddie et al. 1956; Stimson et al. 2009; Hughes et al. 2012). A branch of the superficial epigastric vein on the anterior abdominal wall

was cannulated for adipose tissue sampling (Karpe et al. 2002). A 20G single lumen paediatric central venous catheter (Careflow single lumen catheter, Argon Medical, London, UK) was inserted using the Seldinger technique with the aid of a filtered red light (Frayn et al. 1989; Hughes et al. 2012). Sampling cannulae were kept patent with a slow infusion of 0.9% Saline. Infusions were stopped and a dead space was discarded before blood samples were obtained. A final anterograde 20G cannula was positioned in the opposite antecubital fossa for the intravenous infusion of D2-cortisone and bolus of potassium canrenoate.

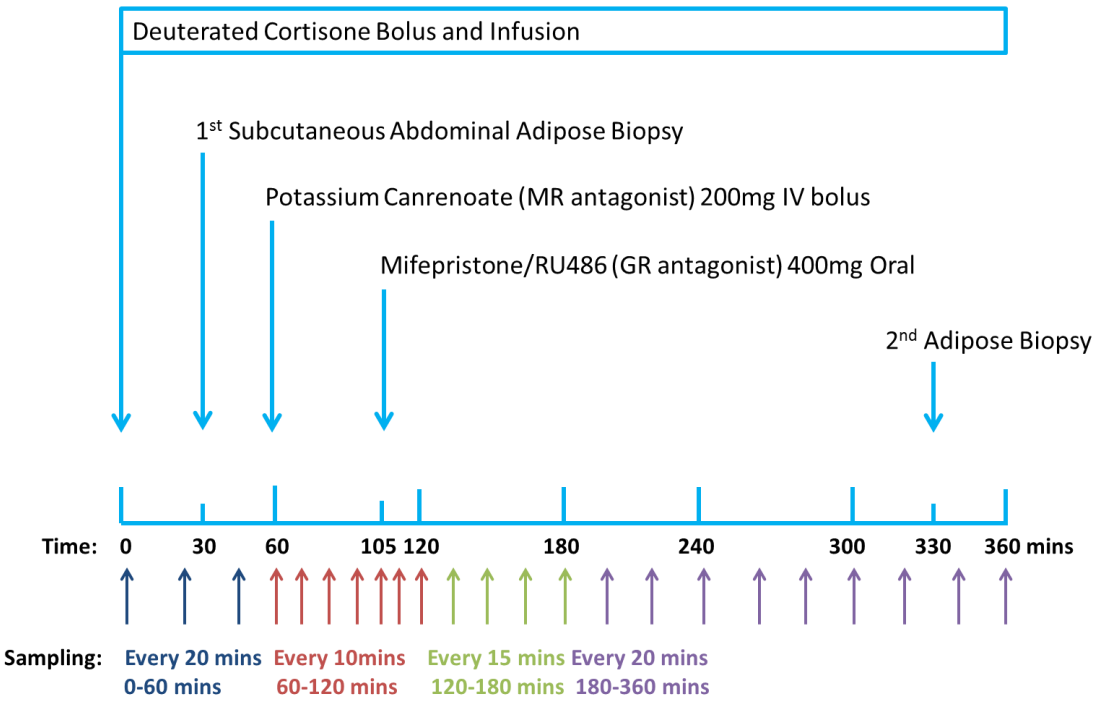
Figure 4-2: Participant set up



At t=0 minutes, an intravenous bolus of 0.076mg D2-cortisone diluted in 20 mL sodium chloride 0.9% *w/v* was given over 5 minutes followed by an infusion at 0.1053 mg/hr for 355 minutes (Figure 4-3). At t+15 minutes, ^{133}Xenon gas was injected subcutaneously lateral to the umbilicus in the abdomen and a gamma counter

(GMS411 Mediscint gamma counter, John Caunt Scientific Ltd, Bury, UK) secured over the area to measure gamma radiation emission (see section 4.2.5.4.1). At t+30 minutes, an abdominal adipose tissue biopsy was performed (see section 4.2.5.5). At t+60 minutes, participants were given an intravenous bolus of MR antagonist potassium canrenoate (Aldactone, Boehringer Ingelheim, Ingelheim, Germany) 200mg over 5 minutes. At t+105 minutes, GR antagonist mifepristone/RU486 (Mifegyne, Exelgyn, Paris, France) 400mg was taken orally with 100 mL of water. A further abdominal fat biopsy was taken at t+330 minutes. At t+360 minutes following the adipose tissue biopsy, the D2-cortisone infusion was stopped, all cannulae removed, participants were given lunch and allowed home.

Figure 4-3: Study diagram



4.2.5.2 Blood sampling protocol

Plasma lithium heparin samples were taken from 3 sites: arterialised (4.9mL), skeletal muscle (4.9mL) and adipose tissue (2.7mL) to measure cortisol, corticosterone, cortisone, 11-dehydrocorticosterone and D2-cortisone. Samples were collected at baseline and every 20 minutes until potassium canrenoate was given at 60 minutes. Thereafter sampling was every 10 minutes for 60 minutes, then every 15 minutes for the next 60 minutes and finally every 20 minutes until the study finished at 360 minutes (Figure 4-3). In addition, canrenoate and mifepristone levels were measured every hour in the arterialised samples. Potassium EDTA samples were obtained to measure ACTH from the arterialised cannula at baseline then every 60 minutes.

4.2.5.3 Sample collection and processing

Samples were obtained in plasma lithium heparin (2.7 and 4.9mL) and potassium EDTA tubes (2.6mL, both Monovette®, Starstedt, Numbrecht, Germany). Samples were gently mixed by inverting several times. Plasma samples were kept on wet ice and subject to centrifugation at 2000 g for 10 minutes at 4 °C within 60 minutes of sampling. Potassium EDTA samples were subject to centrifugation at 2500 g for 10 minutes at 4 °C immediately after sampling. All samples were separated and stored at -80°C until analysis.

4.2.5.4 Blood flow measurements

Blood flow was measured in adipose tissue and skeletal muscle to allow tissue specific *in vivo* kinetic calculations.

4.2.5.4.1 Adipose tissue blood flow

^{133}Xenon wash out was used to measure blood flow in abdominal subcutaneous adipose tissue (Karpe et al. 2002). A dose of 2 MBq was injected subcutaneously approximately 5 cm lateral to the umbilicus and a gamma counter (GMS411 Mediscint gamma counter, John Caunt Scientific Ltd, Bury, UK) was secured in place at the injection site. Measurements of activity were recorded every 20 seconds throughout the study.

Adipose tissue blood flow (ATBF) is calculated from the semilog plot of disappearance of counts versus time in 20 second intervals. For the calculation of ATBF, the relative solubility of ^{133}Xenon between tissue and blood (partition coefficient or λ) is assumed to be 10 ml/g (Goossens & Karpe 2008). ATBF was calculated using the following equation:

Equation 4-1: ATBF (ml/100g tissue/min) =

slope of semilog plot (ln counts/s) x λ (ml/g) x 100 (g) x 60 (s)

4.2.5.4.2 Skeletal muscle blood flow

Skeletal muscle forearm blood flow (FBF) was measured using venous occlusion plethysmography (Hokanson et al. 1975; Rojek et al. 2007; Wilkinson & Webb 2001). For all blood flow measurements, the arm was supported on foam blocks at the elbow and wrist. A mercury-in-silastic strain gauge was applied across the mid forearm and blood flow was obstructed at the wrist using a cuff inflated to 200 mmHg. A further cuff was placed around the upper arm and rapidly inflated to 50 mmHg. Intermittent inflation every 10 seconds for 10 seconds followed by release of venous outflow above

the forearm resulted in dilation of the forearm which was detected by the strain gauge. This corresponds to arterial blood flow rate.

Calibration of the strain gauge was performed prior to each study visit so that 1% change in length of the gauge was equal to 1% change in limb volume. FBF (ml/100 mL tissue/min) was calculated from the slope of the voltage-time curve from the strain gauge using LabChart Reader (Version 8) software (AD Instruments, Oxford, UK). At least 3 measurements were taken hourly and mean flow rate calculated.

4.2.5.5 Biopsy of subcutaneous abdominal fat

Two biopsies of subcutaneous abdominal adipose tissue were obtained at t+30 and t+330 minutes using an aseptic technique from a site lateral to the umbilicus. Local anaesthetic was injected (5mL of 2% lignocaine, Hameln Pharmaceuticals, Gloucester, UK) and a 14G 2.1x80mm needle (Braun, Sheffield, UK) with a 30mL syringe attached was inserted subcutaneously, directed towards the umbilicus. The plunger was withdrawn to create a vacuum and held in place with a 13 x 100 mm glass test tube. The sample of adipose tissue was collected in the syringe and placed on autoclaved aluminium mesh before washing with 0.1% DEPC treated water (section 2.2.2). The sample was then placed in a 2 mL Eppendorf on dry ice before storage at -80°C prior to analysis for glucocorticoid responsive gene expression by qRT-PCR (section 2.8). Up to 3 passes were attempted on each occasion. Samples were not obtained from one subject in whom the procedure was deemed unsafe due to low adiposity.

4.2.6 Sample analysis

4.2.6.1 Preparation of standard curves

Standard curves were prepared for analytes in Table 4-1 as described in section 2.3.2.

Table 4-1: Standard curve analytes and internal standards

Analyte	Standard Curve Range (ng)	Internal Standard	Internal Standard amount (ng)
Cortisol	0.01-400	D4-cortisol	50
Cortisone	0.01-400	D4-cortisol	50
Corticosterone	0.001-40	Epi-corticosterone	10
11-dehydrocorticosterone	0.001-40	Epi-corticosterone	10
D2-cortisone	0.001-40	D4-cortisol	50
Canrenone	0.01-400	D6-canrenone	50
RU486	0.01-400	Alfaxolone	50

Three standard curves were prepared for each analysis. An aqueous curve was used to quantify endogenous glucocorticoids. A separate aqueous D2-cortisone standard curve was prepared to avoid interference between endogenous and deuterated cortisone. A plasma standard curve was prepared for the drug analysis. Standards were made up to 400 µL with stock plasma instead of water.

4.2.6.2 Extraction of plasma samples

Plasma samples were extracted as described in section 2.3.3.

4.2.6.3 LC-MS/MS analysis

Samples were analysed as described in section 2.3.6.2. In addition to the analytes in Table 2-4, the mass transitions, retention times and collision energies were assessed for the following analytes (Table 4-2).

Table 4-2: Chromatographic conditions for LC-MS/MS

Analyte	Mass transition of protonated ion (m/z)	Retention Time (mins)	De-clustering potential (V)	Collision Energy (V)	Collision Cell Exit Potential (V)
D2-Cortisone	363.1→165.1	3.75	166	31	22
Canrenone	341.1→107	6.42	130	47	10
RU486	430.2→134	5.56	130	47	10
D6-Canrenone	347.2→107	6.42	126	43	8
Alfaxolone	333→297	6.29	136	18	10

4.2.7 Data analysis

4.2.7.1 Tracer kinetics

The tracer D2-cortisone was used to allow measurement of flux of endogenous glucocorticoids (or 'tracees') cortisol and corticosterone. The tracer: tracee ratio (TTR) (Equation 4-2) was used for compartmental modelling calculations and allowed assessment of whether endogenous tracees were in steady state. The clearance of both tracer and tracee should be similar in order to calculate whole body rate of appearance. This was calculated for D2-cortisone using Equation 4-3 and compared to previously published clearance data for cortisol and corticosterone.

***Equation 4-2*: Tracer: tracee ratio**

$$TTR\ Cortisol = \frac{D2\ Cortisone}{Cortisol}$$

$$TTR\ Corticosterone = \frac{D2\ Cortisone}{Corticosterone}$$

***Equation 4-3*: Clearance of D2 Cortisone**

$$Clearance = \frac{Rate\ of\ Infusion\ (nmol/\text{min})}{Steady\ State\ Concentration\ \ (nmol/L)}$$

Rate of appearance (Ra) of cortisol and corticosterone were calculated by dividing the rate of infusion of tracer by the corresponding TTR (Wolfe & Chinkes 2005). Whole body rate of appearance of glucocorticoids were calculated using both steady state (Equation 4-4 and Equation 4-5) and non-steady state (Equation 4-6 and Equation 4-7) equations as potassium canrenoate and RU486 were given prior to this.

In order to calculate steady state rate of appearance, tracer and tracee clearance must be similar as these are usually cancelled out. Any differences in clearance between tracees, cortisol and corticosterone, and D2 cortisone were therefore corrected. Clearance of D2 cortisone has been reported as 1.04±0.80 L/min (Hughes et al. 2012) and clearance of cortisol has been relatively consistently reported at 0.28-0.33 L/min (Andrew et al. 2002; Stimson et al. 2007). Clearance of corticosterone in humans has not been formally assessed using tracers however clearance of D8 corticosterone in healthy volunteers was determined by our group in both bolus and steady state infusions (1.46±0.36 and 1.11±0.20 L/min respectively) (Mackenzie 2015). Cortisol clearance is approximately 4-fold slower than D2 cortisone and this was corrected

using Equation 4-4. The clearance rates of corticosterone and D2 cortisone were similar therefore the rate of appearance of corticosterone was calculated without any correction in Equation 4-5.

***Equation 4-4*: Whole body Ra of Cortisol (Steady State)**

$$Ra\ Cortisol\ (nmol/\text{min}) = \frac{Rate\ of\ D2\ Cortisone\ infusion\ (nmol/\text{min})}{TTR\ cortisol * 4}$$

***Equation 4-5*: Whole Body Rate of Appearance of Corticosterone (Steady State)**

$$Ra\ Corticosterone\ \ nmol/min = \frac{Rate\ of\ D2\ Cortisone\ infusion\ (nmol/\text{min})}{TTR\ corticosterone}$$

Non-steady state calculations are derived from the Steele equation first published in 1959 to address the effect of insulin on glucose (Steele 1959). This was applied using Equation 4-6 and Equation 4-7, where F = tracer infusion rate, pV = fractional volume of distribution, t = time, [Cortisol$_t$] = arterialised cortisol concentration at time = t and TTR$_t$ = tracer:tracee ratio at time = t. F was 4.84 nmol/min and pV was assumed to be 12 litres, as has been widely used for glucose (Gastaldelli et al. 1999). This value has also been used in calculating non-steady state rate of appearance of cortisol (Andrew et al. 2005).

***Equation 4-6*: Whole Body Rate of Appearance of Cortisol (Non-Steady State)**

$$Ra\ Cortisol\ (nmol/\text{min}) = \frac{F - pV\ x\ ([Cortisol_{t1}] + [Cortisol_{t2}]/2) \times (TTR_{t2} - TTR_{t1}/(T2 - T1)}{(TTR_{t1} + TTR_{t2}/2)}$$

***Equation 4-7*: Whole Body Rate of Appearance of Corticosterone (Non-Steady State)**

$$Ra\ Corticosterone\ (nmol/\text{min}) = \frac{F-pV\ x\ ([Corticosterone_{t1}]+[Corticosterone_{t2}]/2)\times(TTR_{t2}-TTR_{t1}/(T2-T1)}{(TTR_{t1}+TTR_{t2}/2)}$$

The rate of appearance of cortisol and corticosterone across tissues (skeletal muscle and adipose tissue) was calculated using arteriovenous differences in TTR whilst factoring in blood flow rate through the tissue (Equation 4-8 and Equation 4-9).

***Equation 4-8*: Ra Cortisol across tissue**

$$Ra\ Cortisol\ across\ tissue\ (pmol/100g\ \ tissue/min)$$
$$= (Blood\ Flow\ (BF)x[Cortisol_{Artery}]x\frac{TTR_{Artery}}{TTR_{Tissue}}) - BFx[Cortisol_{Artery}]$$

***Equation 4-9*: Ra Corticosterone across tissue**

$$Ra\ Corticosterone\ across\ tissue\ (pmol/100g\ \ tissue/min)$$
$$= (BFx[Corticosterone_{Artery}]x\frac{TTR_{Artery}}{TTR_{Tissue}}) - BFx[Corticosterone_{Artery}]$$

Net balance was calculated for each glucocorticoid across adipose tissue and skeletal muscle to demonstrate either net release or uptake within the tissue. This was demonstrated by calculating the difference in arterial and tissue glucocorticoid concentration and controlling for blood flow (Equation 4-10 and Equation 4-11).

***Equation 4-10*: Net Balance of Cortisol across tissue**

$$Net\ Balance\ (pmol/100ml\ tissue/min)$$
$$= \left[[Cortisol_{Tissue}] - \left[Cortisol_{Artery}\right]\right] x\ Blood\ Flow(BF)$$

***Equation 4-11*: Net Balance of Corticosterone across tissue**

$$Net\ Balance\ (pmol/100ml\ tissue/\text{min}) = [[Corticosterone_{Tissue}] - [Corticosterone_{Artery}]]\ x\ BF$$

4.2.7.2 Statistical analysis

All data are mean ± SEM unless otherwise stated. Data were analysed using Graph Pad Prism® (version 6.01) and checked for normality of distribution using the Kolmogorov-Smirnov test. If not normally distributed, data were log transformed to achieve this before analysis. Differences between placebo and probenecid groups were assessed using repeated measures analysis of variance (ANOVA) with post-hoc Bonferroni testing. Due to difficulties with sampling from the multiple sampling sites, there were a number of individuals with missing data points. Where these were minimal, specific time points were excluded in the analyses. When multiple data points were missing, data were combined by calculated average values for each subject in three time periods before statistical analysis: 1) pre-drug (t=0-60 min); 2) following potassium canrenoate (t=70-105 min); 3) following potassium canrenoate plus RU486 (t=110-360 min). $P<0.05$ was considered significant.

mRNA transcript levels in adipose tissue were expressed in relation to the abundance of two housekeeping genes (see section 2.8.7.3). Data were compared using two-way ANOVA.

4.3 Results

4.3.1 Characteristics of study participants

Characteristics of all participants are summarised in Table 4-3 below.

Abdominal adipose vein cannulation was technically challenging and was unsuccessful on a number of study visits. In total, 7 patients had adipose tissue data available from both study visits. Arterialised and skeletal muscle data are presented from all 14 participants while adipose tissue data are presented from those 7 participants with data from both visits.

Table 4-3: Participant demographic data

Data are mean ± standard deviation and range for n=14 participants (all male).

	Mean ± standard deviation	**Range**
Age (years)	28.7 ± 13.5	18.0-61.0
BMI (kg/m^2)	24.1 ± 2.6	19.5-30.0
Bioimpedance:		
% Fat	13.9 ± 6.3	6.8-29.3
Mass (kg)	11.2 ± 6.4	5.1-28.7
Blood Pressure:		
Systolic	136.5 ± 6.5	118.0-147.0
Diastolic	81.1 ± 8.1	68.0-97.0
Waist: Hip Ratio	0.9 ± 0.1	0.8-1.0

4.3.2 Whole body glucocorticoid measurements

4.3.2.1 Arterialised glucocorticoid measurements

Arterialised plasma cortisol and corticosterone concentrations are presented in Figure 4-4. During the placebo phase, both cortisol and corticosterone concentrations increased after potassium canrenoate administration and fell steadily thereafter, including after the addition of RU486. Probenecid potentiated the increase in cortisol after canrenoate and after RU486 administration ($p=0.01$). Probenecid tended to increase corticosterone levels following RU486 ($p=0.08$).

4.3.2.2 Whole body rate of appearance of glucocorticoids

Plasma D2 cortisone concentrations and calculated clearance (using Equation 4-3) are shown in Figure 4-5. D2 cortisone reached steady state at 165 minutes. Clearance was unchanged over time once steady state was achieved and there was no difference between drug phases at any point ($p=0.40$).

Figure 4-4: Arterialised cortisol and corticosterone concentrations

Data are mean ± SEM for A) plasma cortisol (blue lines) and B) corticosterone (red lines) concentrations in arterialised samples at time points 0-360 minutes during placebo (unbroken lines) and probenecid phases (dashed lines) (n=14). Comparison was tested by repeated measures ANOVA. Canrenoate (K Can) and mifepristone (RU486) increased cortisol concentrations (p<0.001 vs time) and probenecid significantly increased cortisol compared to placebo (p=0.05 vs placebo, p=0.01 interaction of drug and time). Canrenoate and RU486 increased corticosterone concentrations (log transformed, p<0.001 vs time) while probenecid tended to increase corticosterone (p=0.08 vs placebo, p=0.22 interaction of drug and time).

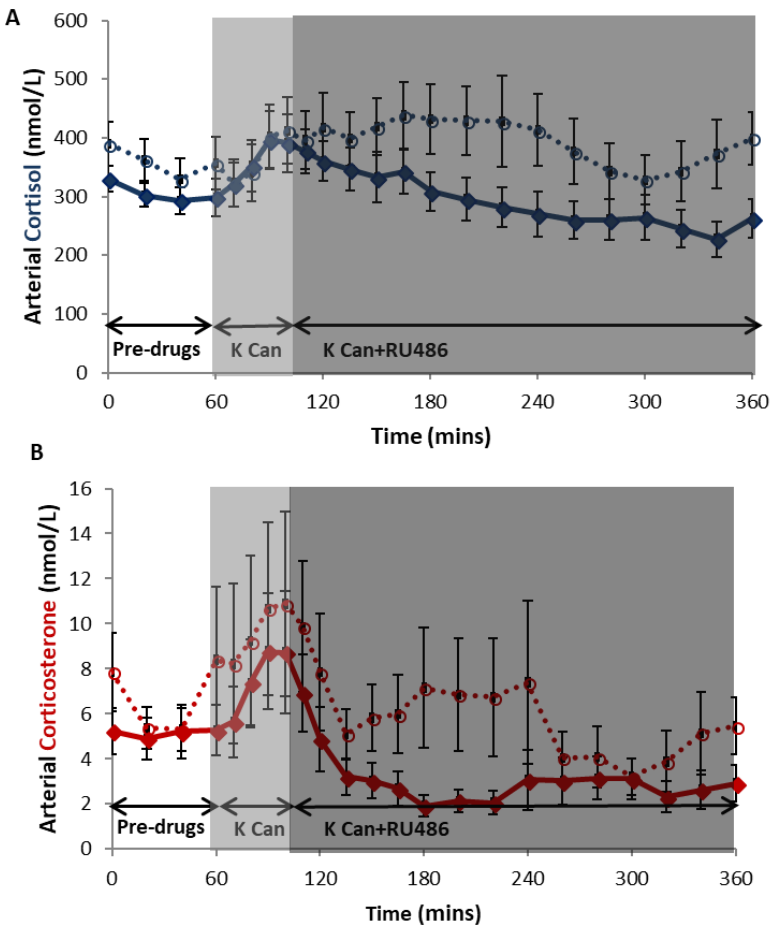
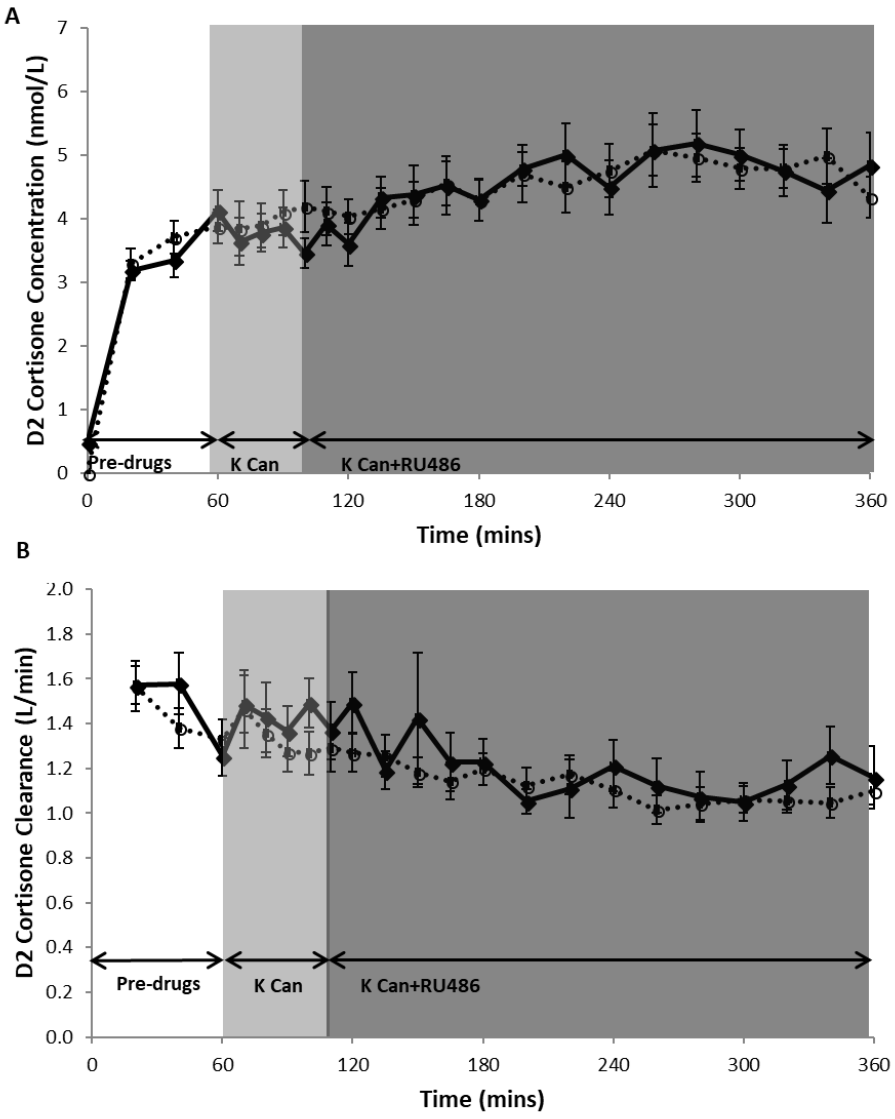


Figure 4-5: Arterialised D2 cortisone plasma concentration and clearance

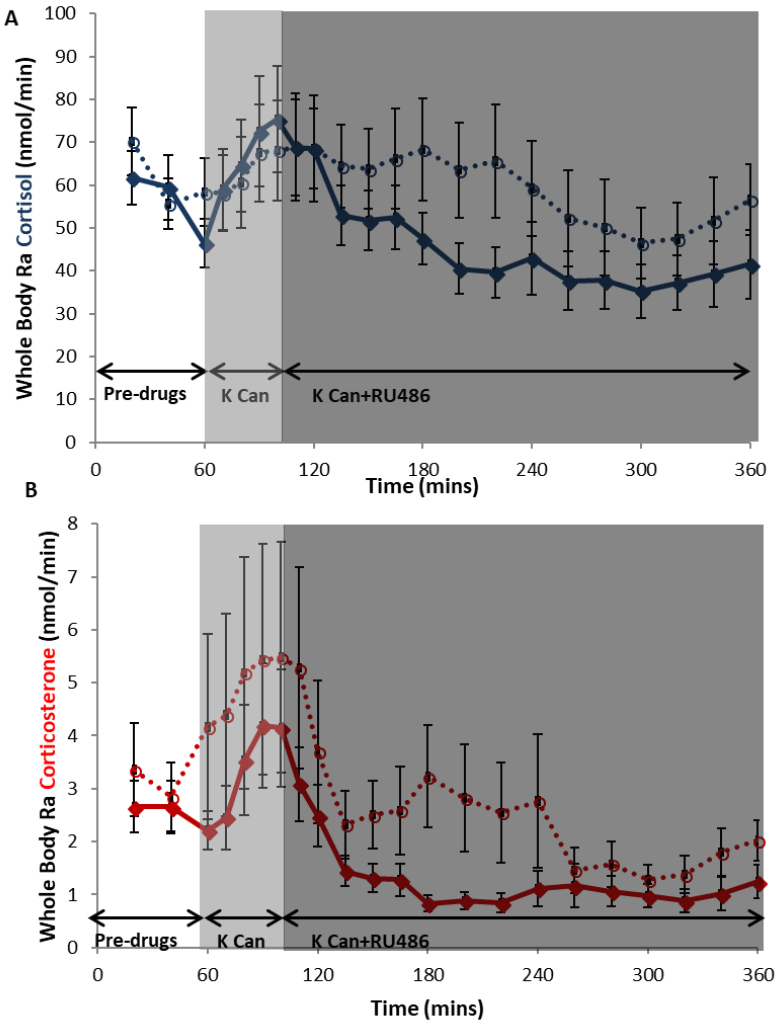
Data are mean ± SEM for A) plasma D2-cortisone concentration and B) clearance of D2-cortisone in placebo (black unbroken line) and probenecid (black dashed line) phases (n=14). Comparison was tested by repeated measures ANOVA. Steady state plasma concentration was achieved from 165 minutes with no significant difference thereafter against time (p=0.08 vs time) and no difference between placebo and probenecid phases (p=0.59 vs placebo). Clearance was unchanged with time or drug at steady state (p=0.11 vs time, p=0.40 vs placebo).



Whole body rate of appearance of glucocorticoids were calculated using both steady state (Equation 4-4 and Equation 4-5) and non-steady state (Equation 4-6 and Equation 4-7) equations as potassium canrenoate and RU486 were given prior to steady state of D2 cortisone being achieved. There was no difference between steady state and non-steady state results and the steady state data is presented in Figure 4-6. There was significant appearance of both cortisol and corticosterone in the placebo phase with peaks after potassium canrenoate infusion ($p<0.0001$ vs zero). Probenecid significantly increased the rate of appearance of cortisol after RU486 administration at t=200 minutes ($p=0.005$). Probenecid did not affect the rate of appearance of corticosterone ($p=0.12$).

Figure 4-6: Whole body rate of appearance of cortisol and corticosterone

Data are mean ± SEM for whole body rate of appearance of A) cortisol (blue lines) and B) corticosterone (red lines) for placebo (unbroken line) and probenecid (dashed line) at time points 0-360 minutes (n=14). Comparison was tested by repeated measures ANOVA. There was significant release of both cortisol and corticosterone (log transformed) during the placebo phase after potassium canrenoate (K Can) (both p<0.001 vs time). In the probenecid phase, there was significantly increased release of cortisol (p=0.005 interaction of drug and time, t=200mins on post hoc Bonferroni testing) but not corticosterone (p=0.12 vs placebo) compared to placebo.

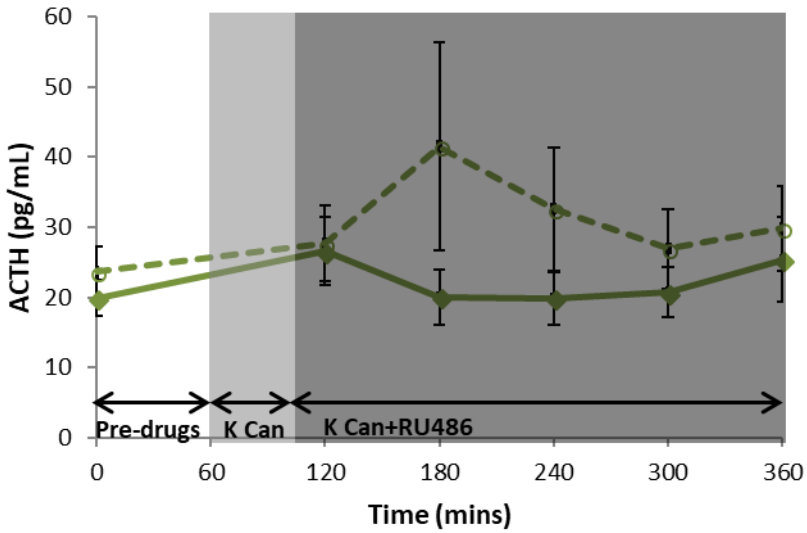


4.3.2.3 ACTH

Plasma ACTH in both placebo and probenecid phases is shown in Figure 4-7. In the placebo phase, there was no significant change in ACTH with time (p=0.22). Probenecid did not significantly alter ACTH concentration but there was a trend for increased ACTH release (p=0.06).

Figure 4-7: Plasma ACTH concentration

Data are mean ± SEM for plasma ACTH concentrations in placebo (green unbroken line) and probenecid phase (green dashed line) (n=14). Comparison was made with repeated measures ANOVA. There was no significant change in ACTH with time but there was a trend for increased ACTH release in the probenecid phase (log-transformed, p=0.22 vs time, p=0.11 vs placebo, p=0.05 interaction of drug and time).



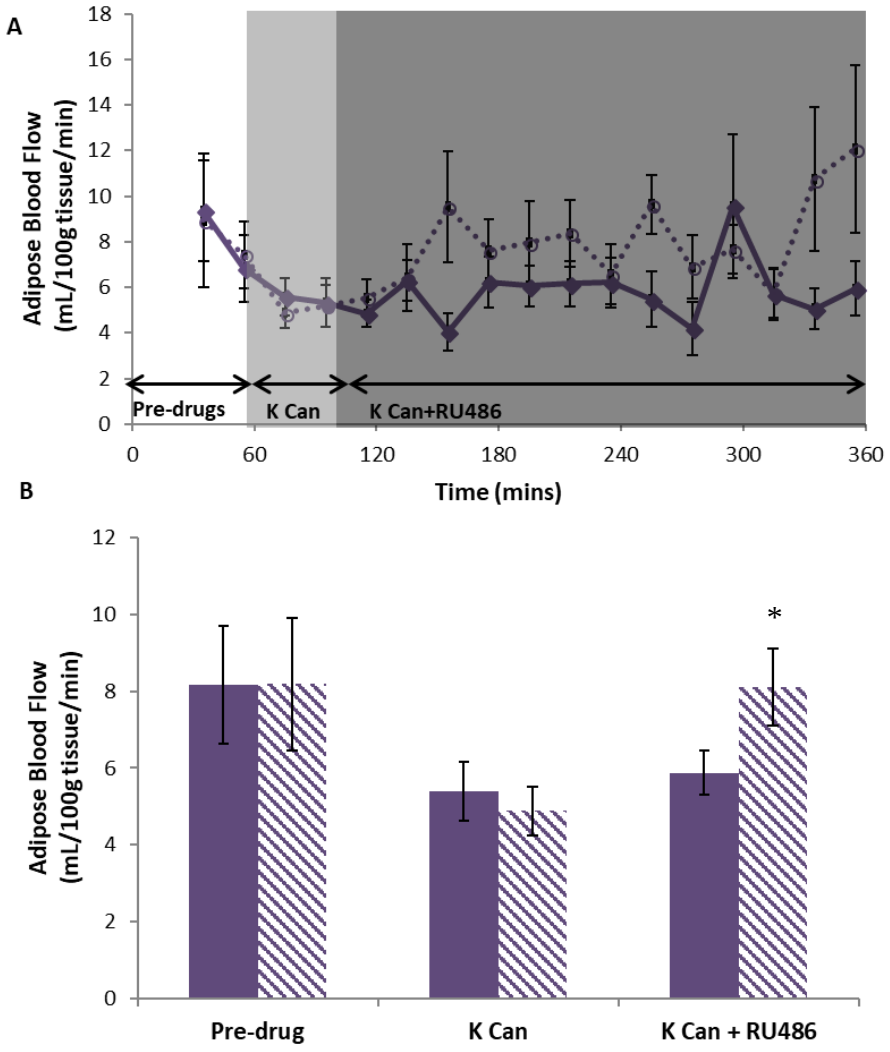
4.3.3 Adipose tissue

4.3.3.1 Adipose tissue blood flow

Due to difficulties with adipose vein cannulation, data presented are for n=7 subjects. Adipose blood flow (calculated as described in section 4.2.5.4.1) is shown at individual time points and was also averaged for each subject for time periods after MR and GR antagonist administration: i.e. pre-drug (0-60 minutes); after potassium canrenoate (70-105 minutes); and after addition of RU486 (110-360 minutes) (Figure 4-8). Data were analysed using groups due to missing data for individual time points. Mean blood flow over the whole study period was 6.1±0.5 and 7.8±0.8 mL/100g tissue/min in the placebo and probenecid phase respectively. There was no significant change in blood flow over time (p=0.12). Probenecid increased adipose tissue blood flow after combined receptor antagonism.

Figure 4-8: Adipose tissue blood flow

*Data are mean ± SEM for adipose tissue blood flow at A) individual time points and B) grouped data: before drugs given (before 60 mins), after potassium canrenoate (70-105 mins) and after RU486 (110-360 mins) during placebo (purple solid line/fill) and probenecid (purple dashed line/striped fill) phases (n=7). Comparison was made using grouped data with repeated measures ANOVA. There was no significant difference over time (p=0.12 vs time) however blood flow significantly increased after combined receptor antagonism in the probenecid phase (p=0.03 interaction of drug and time, *significant on post hoc Bonferroni analysis).*



4.3.3.2 Rate of appearance of glucocorticoids in adipose tissue

Tissue specific rate of appearance of glucocorticoids was measured using the D2-cortisone tracer (using Equation 4-8 and Equation 4-9). There was net uptake of D2-cortisone in adipose tissue in both drug phases and this was not significantly altered by time (Figure 4-9).

Rate of appearance of cortisol and corticosterone in adipose tissue are presented in Figure 4-10. During the placebo phase, there was detectable cortisol production across adipose tissue before, but not after, canrenoate and RU486 were given ($p=0.04$ vs zero). Probenecid did not alter the appearance of cortisol across the tissue.

In the placebo phase, the rate of appearance of corticosterone in adipose was not significantly different to zero either before or after GR and MR antagonism. Probenecid had no significant effect on rate of appearance of corticosterone.

Figure 4-9: Net balance of D2 cortisone in adipose tissue

Data are mean ± SEM for net balance of D2-cortisone in adipose tissue with A) individual time points and B) grouped data during placebo (black unbroken line) and probenecid (grey dashed line) phase (n=14). Comparison was made with repeated measures ANOVA using grouped data. There was significant uptake of D2-cortisone during both placebo and probenecid phases (p=0.002 and p=0.006 vs zero respectively) There was no significant change in uptake after administration of canrenoate or RU486 (p=0.31 v time).

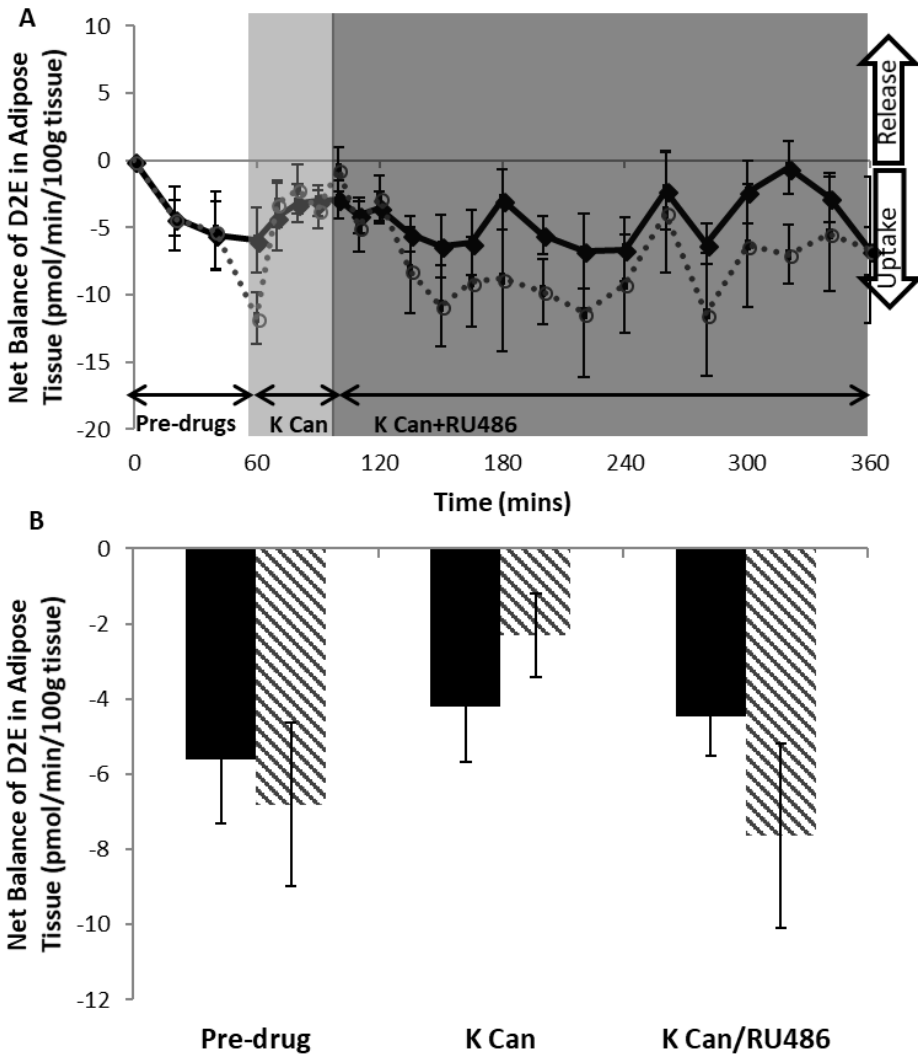
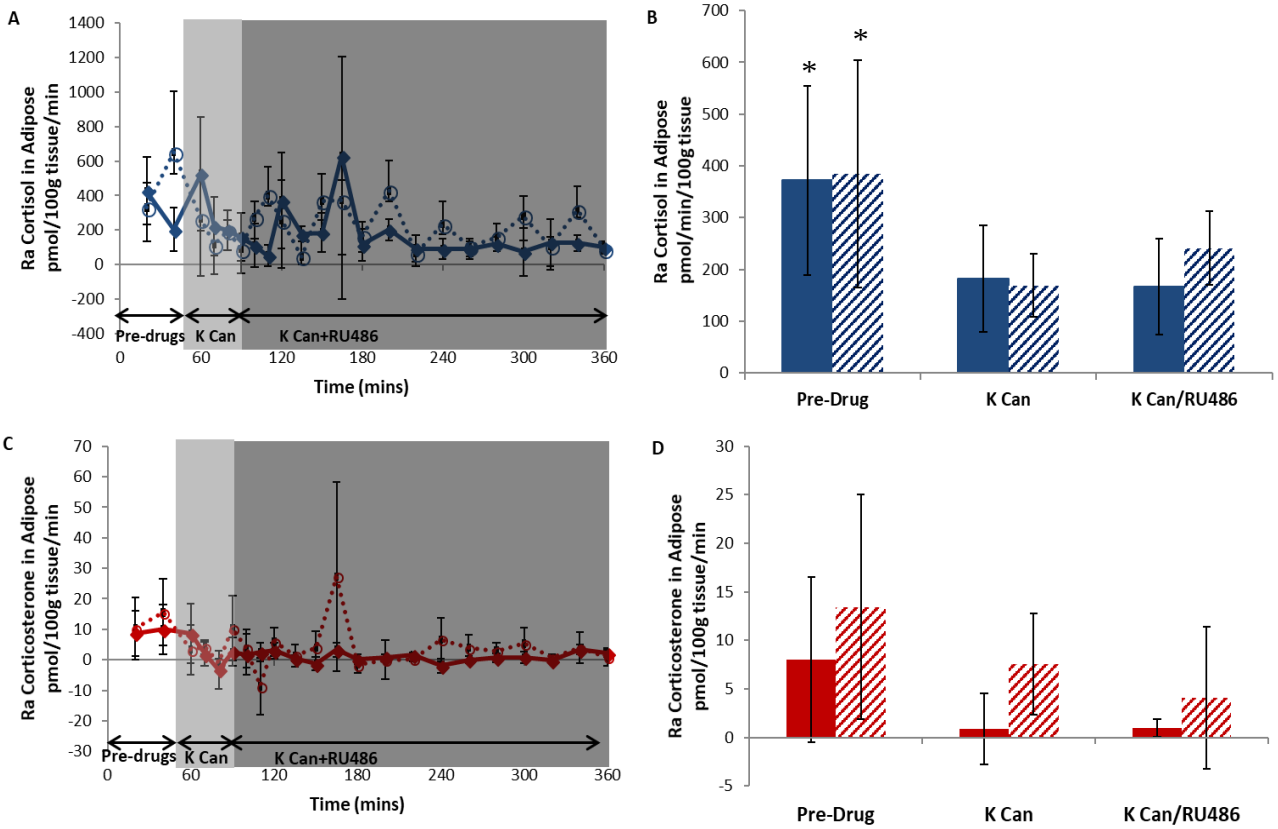


Figure 4-10: Rate of appearance of cortisol and corticosterone in adipose tissue

*Data are mean ± SEM for rate of appearance of cortisol (blue) in adipose tissue using A) individual time points and B) grouped data (before GR/MR antagonism: 0-60mins, after MR antagonism: 60-105mins and after combined receptor antagonism: 110-360mins). Rate of appearance of corticosterone (red) in adipose tissue is shown using C) individual time points and D) grouped data (as above) for placebo (unbroken line/solid fill) and probenecid phase (dashed line/striped fill) (n=7). Comparison was made with repeated measures ANOVA using grouped data. There was significant appearance of cortisol in both placebo and probenecid phases in the pre-drug period compared to zero but not after potassium canrenoate or RU486 (placebo: p=0.05 vs zero, *pre-drug significant on post-hoc Bonferroni testing and probenecid: p=0.04 vs zero, *pre-drug significant on post-hoc Bonferroni testing). There was no significant appearance of corticosterone before or after receptor antagonism in either placebo or probenecid phase (p=0.43 and p=0.21 vs zero respectively).*

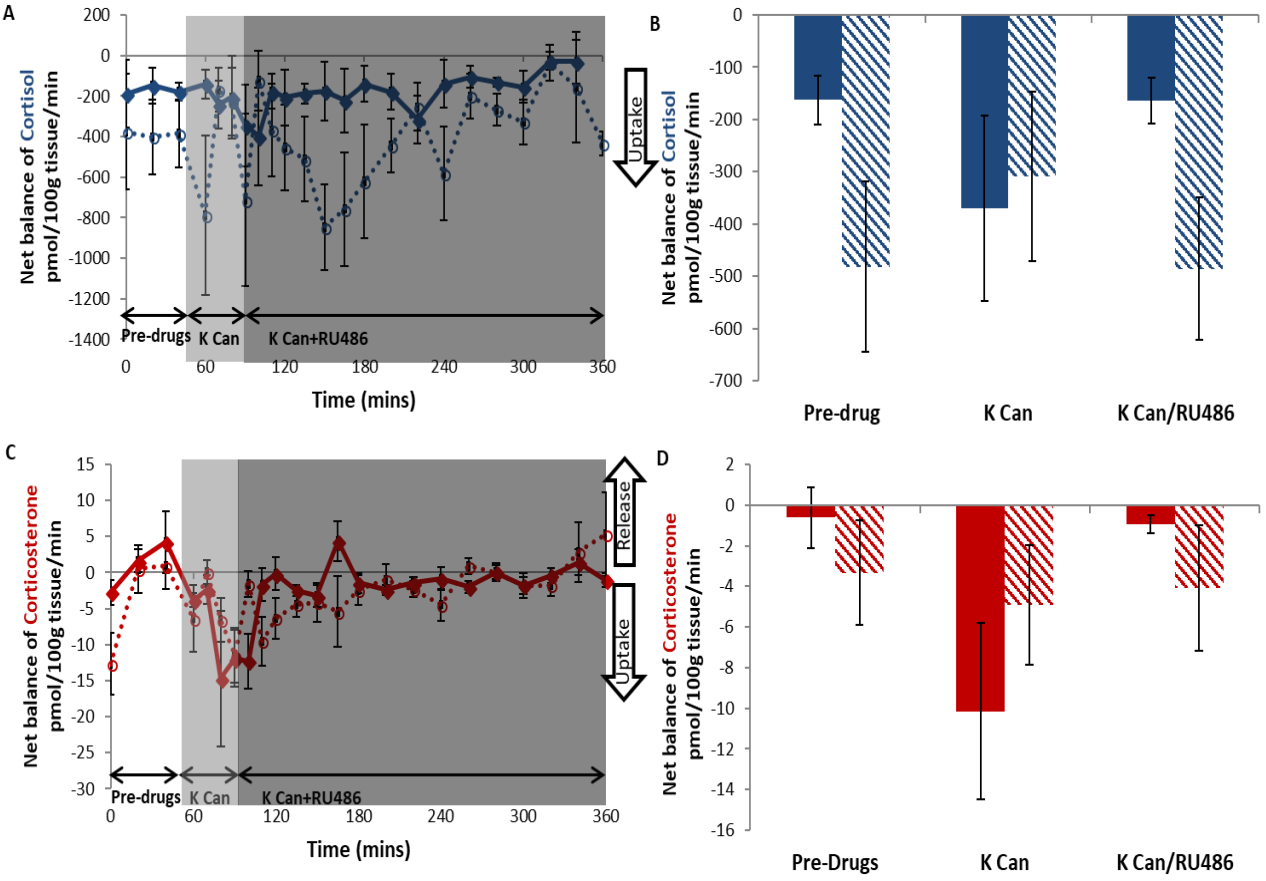


4.3.3.1 Net balance of glucocorticoids within adipose tissue

Net balance of glucocorticoids in adipose was calculated by measuring the arteriovenous difference across the tissue controlled for blood flow and is shown in Figure 4-11 (using Equation 4-10 and Equation 4-11). In the placebo phase, there was detectable uptake of cortisol but not corticosterone in adipose tissue compared with zero ($p=0.01$ and $p=0.13$ v zero respectively). There was no change in uptake of cortisol or corticosterone over time ($p=0.98$ and $p=0.11$ v time respectively). Probenecid had no significant effect on the net balance of either glucocorticoid in adipose tissue.

Figure 4-11: Net balance of cortisol and corticosterone in adipose tissue

Data are mean ± SEM for net balance of cortisol (blue) in adipose tissue using A) individual time points and B) grouped data (before GR/MR antagonism: 0-60mins, after MR antagonism: 60-105mins and after combined receptor antagonism: 105-360mins). Net balance of corticosterone (red) in adipose tissue is shown using C) individual time points and D) grouped data (as above) for placebo (unbroken line/solid fill) and probenecid phase (dashed line/striped fill) (n=7). Comparison was made with repeated measures ANOVA. There was significant uptake of cortisol throughout both placebo and probenecid phases compared to zero but not corticosterone (cortisol: placebo p=0.01 vs zero, and probenecid p=0.02 vs zero, corticosterone: placebo p=0.13 vs zero, and probenecid p=0.14 vs zero). There was no effect over time or when probenecid was compared to placebo for either cortisol or corticosterone (cortisol: p=0.98 vs time, p=0.19 vs placebo and corticosterone: p=0.11 vs time, p=0.92 vs placebo).



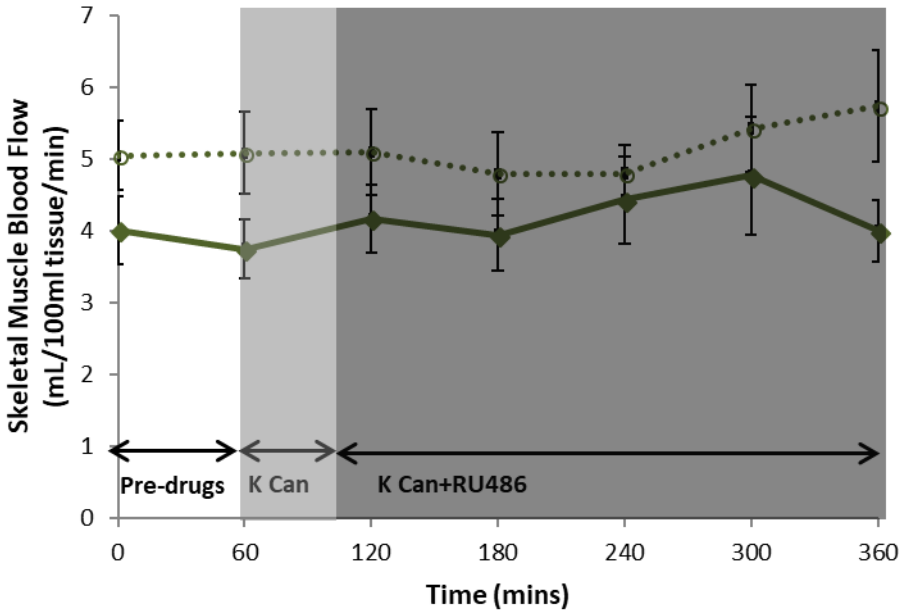
4.3.4 Skeletal muscle

4.3.4.1 Skeletal muscle blood flow

Skeletal muscle blood flow was measured hourly over the study period and is presented in Figure 4-12. In the placebo phase, skeletal muscle blood flow did not change with time (p=0.68). Probenecid increased mean blood flow compared with placebo (5.15±0.48 vs 4.16±0.43 mL/100mL tissue/min respectively, p=0.03).

Figure 4-12: Skeletal muscle blood flow

Data are mean ± SEM for skeletal muscle blood flow at individual time points during placebo (green solid line) and probenecid (green dashed line) phases (n=14). Comparison was made using repeated measures ANOVA. Blood flow was significantly increased during the probenecid phase (p=0.03 vs placebo) and there was no change with time (p=0.68 vs time).

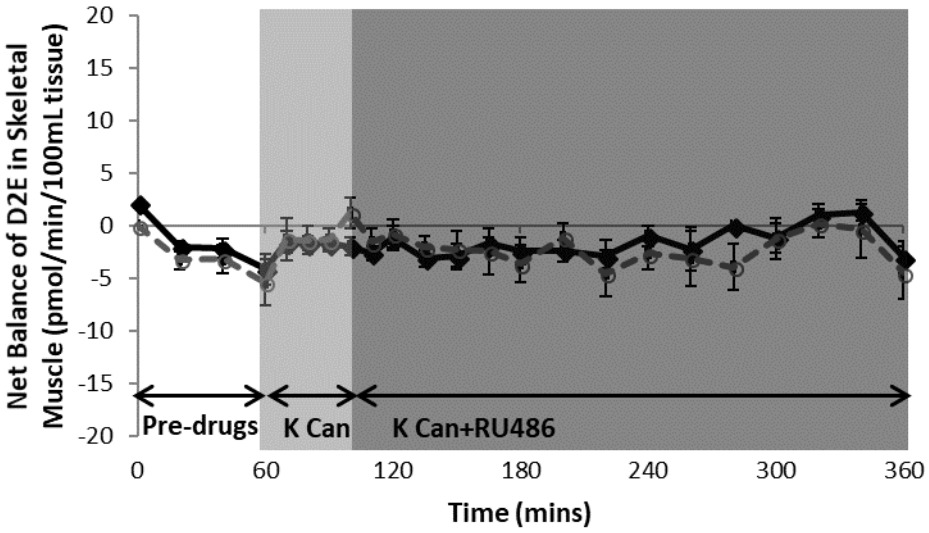


4.3.4.2 Rate of appearance of glucocorticoids in skeletal muscle

There was uptake of D2-cortisone within skeletal muscle which was unaltered by time or drug phase (Figure 4-13).

Figure 4-13: Net balance of D2 cortisone in skeletal muscle

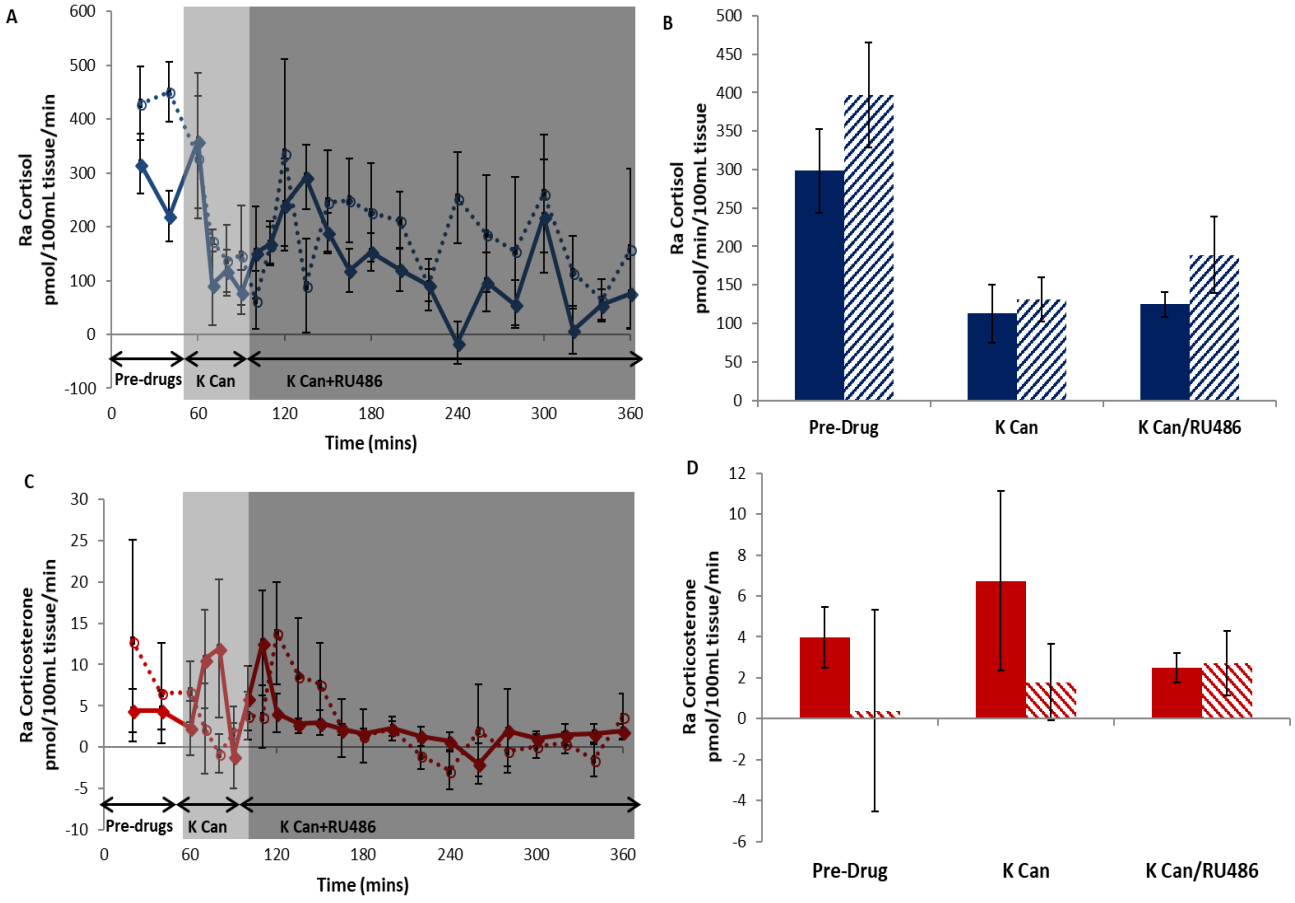
Data are mean ± SEM for net balance of D2-cortisone in placebo (black unbroken line) and probenecid (grey dashed line) phase (n=14). There was significant uptake of D2-cortisone in the placebo phase compared to zero (p=0.005 vs zero) and there was no difference compared with probenecid at any point in the study period (p=0.42 vs time, p=0.64 vs placebo).



Rate of appearance of cortisol and corticosterone are shown in Figure 4-14. In the placebo phase, there was significant detectable appearance of cortisol and corticosterone in skeletal muscle ($p<0.0001$ and $p=0.04$ v zero respectively). There was no significant change in rate of appearance over time ($p=0.09$ and $p=0.07$ vs time respectively). Probenecid did not alter the appearance of cortisol or corticosterone ($p=0.15$ and $p=0.81$ vs placebo respectively).

Figure 4-14: Rate of appearance of cortisol and corticosterone in skeletal muscle

Data are mean ± SEM for rate of appearance of cortisol (blue) in skeletal muscle using A) individual time points and B) grouped data (before GR/MR antagonism: 0-60mins, after MR antagonism: 60-105mins and after combined receptor antagonism: 105-360mins). Rate of appearance of corticosterone (red) in skeletal muscle is shown using C) individual time points and D) grouped data (as above) for placebo (unbroken line/solid fill) and probenecid phase (dashed line/striped fill) (n=14). Comparison was made with repeated measures ANOVA. There was significant appearance of cortisol vs zero in both placebo and probenecid phases throughout the study period (placebo: p<0.0001 vs zero and probenecid: p=0.0002 vs zero) Rate of appearance of corticosterone was also significant compared to zero (placebo: p=0.04 at t=80 and 110 on post-hoc Bonferroni testing, probenecid: p=0.01 at t=20 and 120 on post-hoc Bonferroni testing). There was no significant effect over time or when probenecid was compared to placebo for cortisol or corticosterone (cortisol: p=0.09 vs time, p=0.15 vs placebo and corticosterone: p=0.07 vs time, p=0.81 vs placebo).

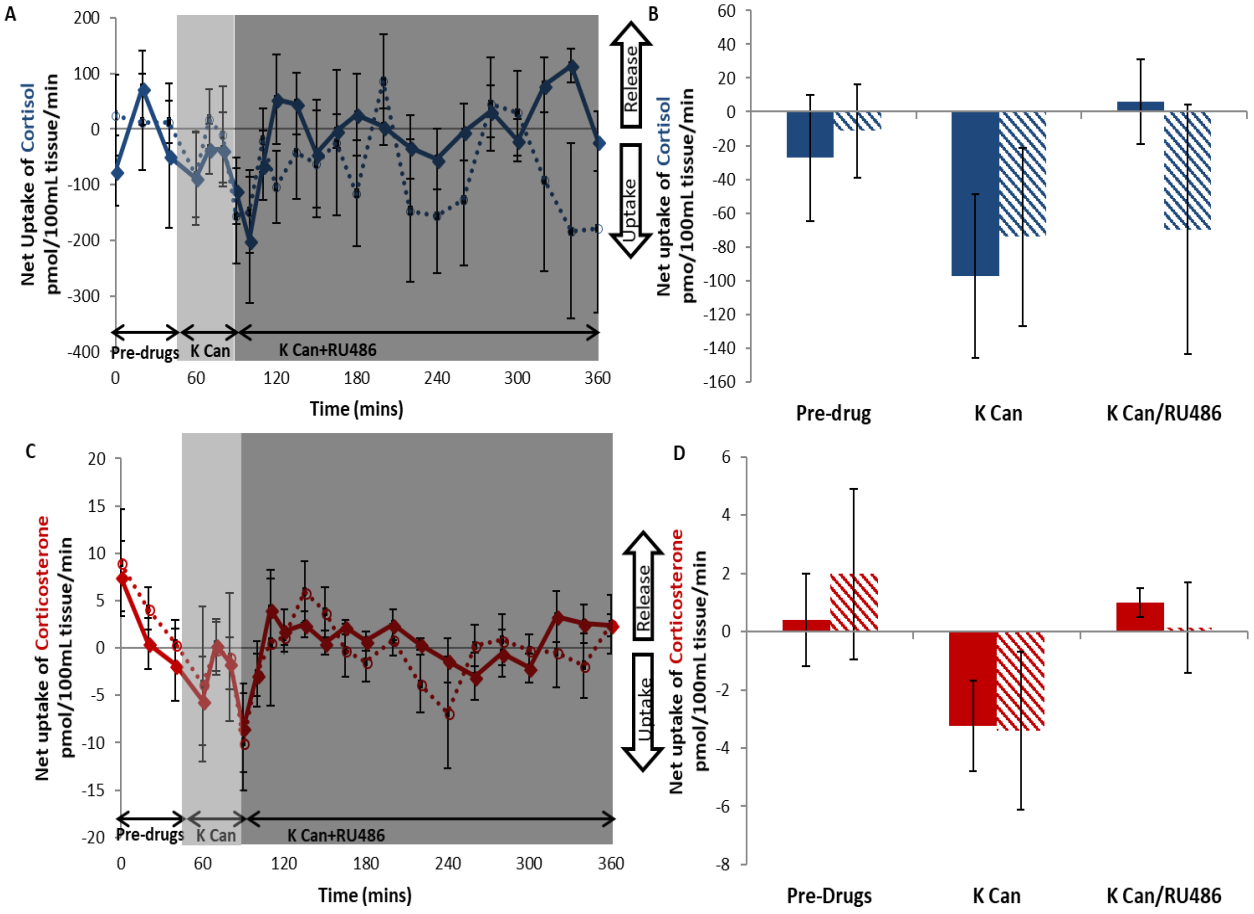


4.3.4.3 Net balance of glucocorticoids in skeletal muscle

Net balance of glucocorticoids in skeletal muscle is shown in Figure 4-15. In the placebo phase, there was no significant detectable uptake or release of cortisol or corticosterone across skeletal muscle ($p=0.40$ and $p=0.39$ vs zero respectively). There was no change in net balance over time. Probenecid had no significant effect on net balance of cortisol or corticosterone within skeletal muscle ($p=0.53$ and $p=0.91$ vs placebo respectively).

Figure 4-15: Net balance of cortisol and corticosterone in skeletal muscle

Data are mean ± SEM for net balance of cortisol (blue) in skeletal muscle using A) individual time points and B) grouped data (before GR/MR antagonism: 0-60mins, after MR antagonism: 60-105mins and after combined receptor antagonism: 105-360mins). Net balance of corticosterone (red) in skeletal muscle is shown using C) individual time points and D) grouped data (as above) for placebo (unbroken line/solid fill) and probenecid phase (dashed line/striped fill) (n=14). Comparison was made with repeated measures ANOVA. There was no significant difference in net balance of cortisol or corticosterone when compared to zero (cortisol: placebo p=0.40 vs zero, and probenecid p=0.27 vs zero, corticosterone: placebo p=0.40 vs zero, and probenecid p=0.79 vs zero). There was no effect over time or when probenecid was compared to placebo for either cortisol or corticosterone (cortisol: p=0.15 vs time, p=0.53 vs placebo and corticosterone: p=0.10 vs time, p=0.91 vs placebo).



4.3.5 Summary of Glucocorticoid Actions

In summary, arterialised cortisol concentration and rate of appearance increased as expected after corticosteroid receptor inhibition and probenecid significantly potentiated this effect. Corticosterone concentration and rate of appearance were similarly increased and there was a trend for probenecid to enhance this effect.

In adipose tissue, there was increased rate of appearance of cortisol and relative uptake within the tissue. Corticosterone showed no increase in rate of appearance or any uptake or release compared to zero. In skeletal muscle, there was an increase in rate of appearance of both cortisol and corticosterone but no significant uptake or release of either. Probenecid had no significant effect on either glucocorticoid.

Table 4-4: Glucocorticoid Summary Table

** Denotes significant change compared to placebo (p<0.05)*

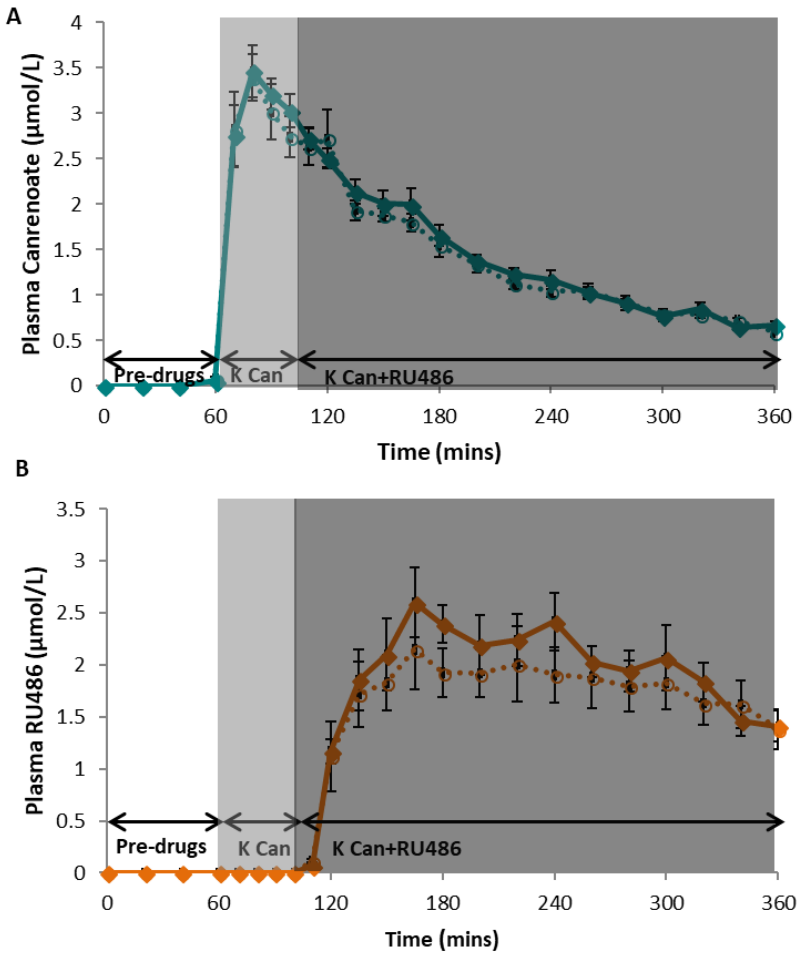
		Cortisol		Corticosterone	
		Placebo	**Probenecid**	**Placebo**	**Probenecid**
Arterial	**Concentration**	↑	↑↑*	↑	↑↑
	Whole Body Rate of Appearance	↑	↑↑*	↑	↑↑
Adipose Tissue	**Rate of Appearance**	↑	No effect	No change	No effect
	Net Balance	Uptake	No effect	No uptake or release	No effect
Skeletal Muscle	**Rate of Appearance**	↑	No effect	↑	No effect
	Net Balance	No uptake or release	No effect	No uptake or release	No effect

4.3.6 Plasma canrenoate and RU486 concentrations

There was no difference in plasma concentrations of potassium canrenoate and RU486 during placebo and probenecid phases (Figure 4-16).

Figure 4-16: Plasma drug concentrations

Data are mean ± SEM for plasma arterialised concentrations of A) canrenoate and B) RU486 during placebo (unbroken line) and probenecid (dashed line) phases (n=14). Comparison was made using repeated measures ANOVA. There was no difference in either canrenoate or RU486 concentration between placebo and probenecid phases (canrenoate: p=0.36 vs placebo, RU486: p=0.28 vs placebo).

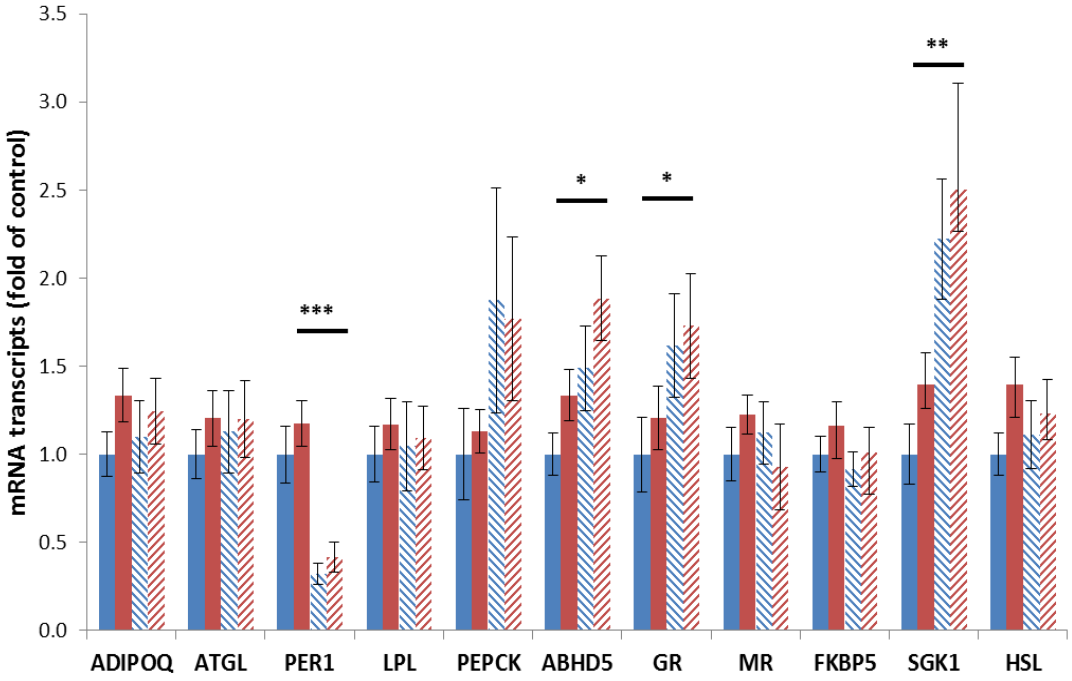


4.3.7 Glucocorticoid-responsive gene expression in subcutaneous adipose tissue

Expression of glucocorticoid responsive genes in subcutaneous adipose tissue are shown inFigure 4-17. Comparison was made between the sample taken at baseline and after combined receptor antagonism in the placebo and probenecid phase. Data for placebo at baseline were normalised to 1 and results are presented as fold of change from baseline. PER1 mRNA transcript levels fell over time ($p<0.001$ and $p<0.05$ respectively). Gene transcripts for ABHD5, GR and SGK1 increased with time ($p<0.05$). Probenecid did not alter mRNA transcript levels.

Figure 4-17: mRNA transcript levels of glucocorticoid responsive genes in subcutaneous adipose tissue

*Data are mean ± SEM for mRNA transcript levels of glucocorticoid responsive genes adiponectin (ADIPOQ), adipose triglyceride lipase (ATGL), period circadian clock (PER1), lipoprotein lipase (LPL), phosphoenolpyruvate carboxykinase (PEPCK), abhydrolase domain containing protein 5 (ABHD5), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), FK506 binding protein 5 (FKBP5), serum and glucocorticoid-regulated kinase (SGK1) and hormone sensitive lipase (HSL) (n=12). Data are expressed as a ratio to the mean of 2 housekeeping genes. Comparisons between first (solid fill) and second (striped fill) biopsy and between placebo (red fill) and probenecid (blue fill) phases were made with two -way ANOVA. PER1 transcripts fell significantly over the study period while ABHD5, GR and SGK1 transcript levels increased. *p<0.05 **p<0.01 ***p<0.001 vs time.*



4.4 Discussion

In a randomised placebo-controlled crossover study of the effects of ABCC1 inhibition on central negative feedback and glucocorticoid occupancy of corticosteroid receptors in adipose tissue and skeletal muscle, we have demonstrated a central effect of probenecid on the HPA axis.

This study is the first to our knowledge to measure the direct effects of ABCC1 inhibition on glucocorticoid flux *in vivo* in humans. In order to achieve ABCC1 inhibition, participants were given probenecid 1 g BID for five days prior to the study visits. Probenecid has historically been used as a synergistic agent to improve the efficacy of some antibiotics and subsequently as a uricosuric agent in the treatment of gout (Cunningham et al. 1981; Robbins et al. 2012). The half-life of probenecid is between 3-8 hours and therapeutic doses range from 0.5-3 g (Selen et al. 1982; Cunningham et al. 1981; Robbins et al. 2012). It has been demonstrated as an ABCC1 inhibitor with no effect on ABCB1 and used in *in vitro* and *in vivo* studies for this purpose (Feller et al. 1995; Gollapudi et al. 1997; Webster & Carlstedt-Duke 2002). We used a dose of 1g twice daily which was taken on the morning of each study visit to ensure ABCC1 inhibition was maintained.

We hypothesised that inhibition of ABCC1 would have no effect on central negative feedback of the HPA axis given the relative abundance of ABCB1 at the blood brain barrier (Reul & de Kloet 1985; Karssen et al. 2001; Raubenheimer et al. 2006). In fact, our results suggest ABCC1 is significant in negative feedback with an enhanced activation of the HPA axis to MR and GR antagonism during ABCC1 inhibition. This

effect appears to be centrally mediated with a trend for increased ACTH release with probenecid. This suggests an increased drive of ACTH induced adrenal production rather than any primary effect on the adrenal or peripheral clearance of glucocorticoids. This may reflect increased occupancy of corticosteroid receptors in the pituitary during probenecid therapy and suggests ABCC1 expression is significant in pituitary driven HPA negative feedback.

The significance of ABCB1 in glucocorticoid transport at the blood brain barrier was highlighted by Karssen et al in 2001 who demonstrated that ABCB1 limited access of cortisol and cortisone to the brain suggesting corticosterone played a more significant role in negative feedback (Karssen et al. 2001). The corticosterone: cortisol ratio was significantly higher in post mortem brain samples compared to plasma however they did not examine specific areas within the brain. It should be noted that there is ABCC1 expression at the blood brain barrier which has been shown to have effects on drug efflux although expression is relatively lower than ABCB1 (Huai-Yun et al. 1998; Tan et al. 2000; Rao et al. 1999).

Expression of ABCB1 and ABCC1 in the pituitary, out with the blood brain barrier, is less well defined. Bernstein et al investigated the vascular and extravascular distribution of ABC transporters in post mortem human brain samples and showed both ABCB1 and ABCC1 were highly expressed in the anterior pituitary (Bernstein et al. 2014). Given that ABCC1 exports drug conjugates of glutathione, leukotrienes and other organic anions, it is not unexpected that it may play a role in protecting the brain from exposure to these toxins (Dean et al. 2001).

Our data has highlighted that ABCC1 likely plays a significant role in regulating access of corticosterone to the pituitary. The data from Karssen et al suggests the higher relative expression of ABCB1 at the blood brain barrier is significant in regulating glucocorticoid access to the hypothalamus and higher limbic structures. The same does not appear to be true of the pituitary where ABCC1 appears to play a more significant role. It should be noted that we have not tested the effects of ABCB1 inhibition on HPA negative feedback and without this data, we cannot conclude which glucocorticoid has the more proportionate effect. If ABCB1 inhibition lead to even greater activation of the axis, our hypothesis of the disproportionate effects of cortisol and corticosterone on negative feedback would still be valid.

We cannot exclude that probenecid may also have inhibitory effects on ABCB1 although this seems less likely. The main body of literature describing probenecid effects on ABC transporters would refute this but one group showed that probenecid reduced both mRNA and protein levels of ABCB1 in human neuroblastoma cells (Campos-Arroyo et al. 2016). This is an isolated report in a human tumour cell line which may not be true of normal human tissues. Our own data from *in vitro* and murine experiments support probenecid as an inhibitor of ABCC1 and not ABCB1 (Nixon et al. 2016).

This study was designed to determine the occupancy of corticosteroid receptors by cortisol and corticosterone in adipose tissue and skeletal muscle in the presence and absence of ABCC1 inhibition. MR and GR antagonists potassium canrenoate and RU486 were given to block respective receptors and displace bound glucocorticoids.

We expected to demonstrate release of glucocorticoids from each tissue in response to these antagonists but in fact, we have evidence of uptake within both tissues. Our adipose tissue data was limited to n=7 due to technical difficulties and was therefore under powered so may have missed any small corticosteroid release. Our skeletal muscle data however was fully powered and displacement was still not demonstrated.

Displacement of glucocorticoids across tissue can be measured, for example, in heart (Iqbal et al. 2014), but the increments were very small in this study and may be difficult to demonstrate in other tissues. We included the infusion of the tracer D2-cortisone to enhance sensitivity however there was no difference with either net balance or rate of appearance data. Detecting displacement from corticosteroid receptors requires those displaced glucocorticoids to exit the cell and enter the local circulation. It is, of course, possible that there has been displacement from GR and MR but these glucocorticoids remained within the cell. It is also feasible that the enhanced central effect on the HPA axis stimulating adrenal release of cortisol and corticosterone may have masked any effect of displacement due to the change in circulating concentrations.

Another factor in the calculation of release and rate of appearance in these tissues was blood flow. Skeletal muscle blood flow was higher than measured in a previous clinical study in healthy individuals (2.8±0.2 mL/min/100g tissue) (Hughes et al. 2012) but similar to a more recent study of lean and obese individuals (Anderson 2017). Probenecid significantly increased skeletal muscle blood flow throughout the study period. In addition to inhibiting ABCC1, probenecid is a potent transient receptor potential vanilloid 2 (TRPV2) agonist, which has a significant role in cardiac function.

Probenecid has been shown to be a positive inotrope and cause increased contractility in a murine model and may therefore affect peripheral blood flow through this mechanism (Rubinstein et al. 2014). In a direct comparison of the effect of probenecid on endothelial function however, forearm blood flow was unaltered compared to placebo after 7 days of 500mg BID (George et al. 2006). Our data would support the increased inotropic effect of probenecid leading to increased skeletal muscle blood flow throughout the probenecid phase.

In adipose tissue, calculated blood flow was similar to the previous clinical study in healthy individuals which was reported as 5.7±1.3 mL/min/100g tissue (Hughes et al. 2012). Probenecid also increased adipose blood flow but not from baseline and only after combined receptor antagonism. This suggests a glucocorticoid-mediated mechanism rather than a direct effect on cardiac output.

Although the difference in blood flow between placebo and probenecid was significant, the calculation for glucocorticoid rate of appearance takes blood flow into account for this reason and therefore is unlikely to be a major confounding factor.

Data from subcutaneous adipose tissue biopsies were somewhat contradictory. Expression of *GR* levels increased 1.5-fold in adipose tissue at the end of the study period which suggests we were successful in antagonising adipose *GR*. The inverse has been shown recently with reduced *GR* and *MR* transcript levels in adipose tissue in the presence of high glucocorticoid concentrations (Stimson et al. 2017). There was no similar effect seen with MR but potassium canrenoate was given first and any compensatory increase may have been transient. The glucocorticoid sensitive gene

PER1 fell significantly with time in both phases. *PER1* encodes period circadian homolog 1, a vital component of the circadian clock in peripheral tissues which is acutely sensitive to glucocorticoids (Stavreva et al. 2009). This may be interpreted as successful inhibition of *GR* but given its circadian nature, we would need to have had placebo controls for canrenoate and RU486 administration to confirm this; unfortunately, this was considered impractical due to the invasive nature of the protocol. The rise in *ABHD5* and *SGK1* transcript levels following receptor antagonism are unexpected as both are glucocorticoid sensitive, the former a co-factor of key lipase *ATGL* and the latter an early transcriptional glucocorticoid target activated by insulin (Lord & Brown 2012; Ullrich et al. 2005). Although *SGK1* is regulated by glucocorticoids, both acute and chronic inflammation are also able to induce expression (Schernthaner-Reiter et al. 2015). Both biopsies were taken from the same area of adipose tissue and inflammation from the initial biopsy may have influenced expression in the subsequent sample. Although we showed no significant effect of probenecid on transcript expression at baseline, there was a trend for expression to be higher in the probenecid phase for all genes tested. These data represent n=12 as one participant had low adiposity and biopsies were not attempted and another had a poor yield from one sample and was therefore excluded from analysis. This study was powered on the basis of measuring differences in glucocorticoid rates of appearance rather than changes in adipose transcript levels and may therefore be under powered to detect any significant difference in the probenecid phase.

Our data represents the first evidence of ABCC1 regulating pituitary driven HPA negative feedback *in vivo* in humans. While we were unable to detect effects in peripheral tissues, our data further highlights the discrete roles of cortisol and corticosterone in humans.

Chapter 5. Corticosterone vs cortisol as glucocorticoid replacement therapy

5.1 Introduction

The transmembrane transporter ABCC1 transports corticosterone but not cortisol and is highly expressed in adipose tissue and skeletal muscle which are important metabolic tissues (Webster & Carlstedt-Duke 2002; Nixon et al. 2016). Conversely, the transporter ABCB1 exports cortisol but not corticosterone and is expressed at the blood brain barrier, leading to accumulation of corticosterone in the brain and CSF (Karssen et al. 2004; Raubenheimer et al. 2006). We have shown in the previous chapter that acute HPA axis feedback may be modulated by ABCC1 at the pituitary. This may have implications in patients requiring glucocorticoid replacement therapy and suppression of the HPA axis drive such as in CAH.

CAH is a common genetic endocrine condition of impaired steroidogenesis most frequently caused by mutation in the *CYP21A2* gene encoding the enzyme 21-hydroxylase (Han et al. 2014). Deficiency in 21-hydroxylase causes disrupted cortisol synthesis and impaired negative feedback of the HPA axis leading to subsequent over-production of 17-OHP and adrenal androgens. Glucocorticoid replacement therapy is given to replace glucocorticoid and suppress androgen over-production. Many patients also have mineralocorticoid deficiency due to 21-hydroxylase playing a key role in aldosterone production. Observational studies have shown significant morbidity and mortality in this patient cohort with high prevalence of cardiovascular disease and osteoporosis (Arlt et al. 2010). Mortality is higher than matched controls and quality of life is poor (Han, Krone, et al. 2013; Falhammar et al. 2014). There is a lack of consensus on optimal management of this condition and current therapies with synthetic glucocorticoids such as dexamethasone are associated with poor health

outcomes without necessarily improving markers of disease control (Han, Stimson, et al. 2013). The most commonly used glucocorticoid treatment is hydrocortisone (Forss et al. 2012) and newer modified release preparations have been developed to try and address these issues however metabolic side effects on insulin resistance and bone are still observed (Mallappa et al. 2015).

The clinical study presented in this chapter aimed to test the hypothesis that corticosterone replacement is equally effective as cortisol at suppressing ACTH and adrenal androgens and has less adverse metabolic side effects in individuals with CAH. We therefore compared the effects of placebo, corticosterone and hydrocortisone infusions on biochemical markers of disease control and circulating metabolic biomarkers *in vivo* in a randomised crossover study. Assessment of the dose-response effect was performed using 2-step ramped steady state infusions of placebo, hydrocortisone and the previously validated stable isotope tracer, 2,2,4,6,6,17α,21,21-$[^2H]_8$-corticosterone (D8-corticosterone) (Mackenzie 2015).

Unfortunately, as is often the case in clinical studies, unavoidable delays lead to the late start of this study. Due to time limitations, I was unable to complete the study before the end of my research period. While at the time of writing this study is ongoing, I present interim data of the 8 participants who have completed the full protocol.

5.2 Methods

5.2.1 Ethical and research governance approvals

This study was approved by the South-East Scotland Research Ethics Committee 02 (16/SS/0045) and NHS Lothian Research and Development committee (2016/0069). Approval was also secured from NHS Greater Glasgow and Clyde Research and Development committee (GN16ME098) to allow recruitment from both health boards due to the small number of patients in Lothian. Research support approvals were secured with the Royal Infirmary of Edinburgh Clinical Research Facility (RIECRF) and NHS Lothian laboratories at the Royal Infirmary of Edinburgh (RIE).

5.2.2 Study design

A double blind randomised crossover study was performed in patients with CAH comparing the biochemical and metabolic effects of corticosterone, cortisol and placebo infusion over 5.5 hours. Participants attended on three occasions after overnight fast, receiving each drug or placebo in random order. Cortisol (hydrocortisone) and D8-corticosterone were infused to achieve 400 then 800 nmol/L to reflect the typical plasma concentration after 5 and 10mg of hydrocortisone replacement (Debono et al. 2009). 6,6-$[^2H]_2$-glucose (D2-glucose) and 1,1,2,3,3-$[^2H]_5$-glycerol (D5-glycerol) were infused to measure glucose metabolism and lipolysis respectively. Blood pressure was measured regularly and pulse wave analysis and velocity were measured during low and high glucocorticoid concentrations. An adipose tissue biopsy was taken at the end of each visit to compare glucocorticoid specific transcripts.

5.2.3 Participants

Participants (n=8, 6 female) were identified from patient databases in Glasgow and Edinburgh and were approached at their clinic appointment by a member of the direct care team or contacted by post and sent the participation information sheet along with a cover letter. Subjects were then invited for a screening visit to discuss the study in detail and to obtain written informed consent. Eligibility was assessed through acquisition of relevant medical history, clinical examination and baseline blood tests.

5.2.3.1 Inclusion criteria

- Aged 18-80 years
- Male or female
- Diagnosed with classic CAH secondary to 21-hydroxylase deficiency
- On glucocorticoid replacement therapy for CAH
- Blood pressure greater than 90/50 mmHg at screening

5.2.3.2 Exclusion criteria

- On additional oral, inhaled or topical glucocorticoids for an unrelated condition
- Abnormal screening bloods (full blood count (FBC), urea and electrolytes (U+E), thyroid function tests (TFTs), liver function tests (LFTs), random glucose)
- Recent admission with adrenal crisis in preceding 6 months

5.2.4 Sample size calculation

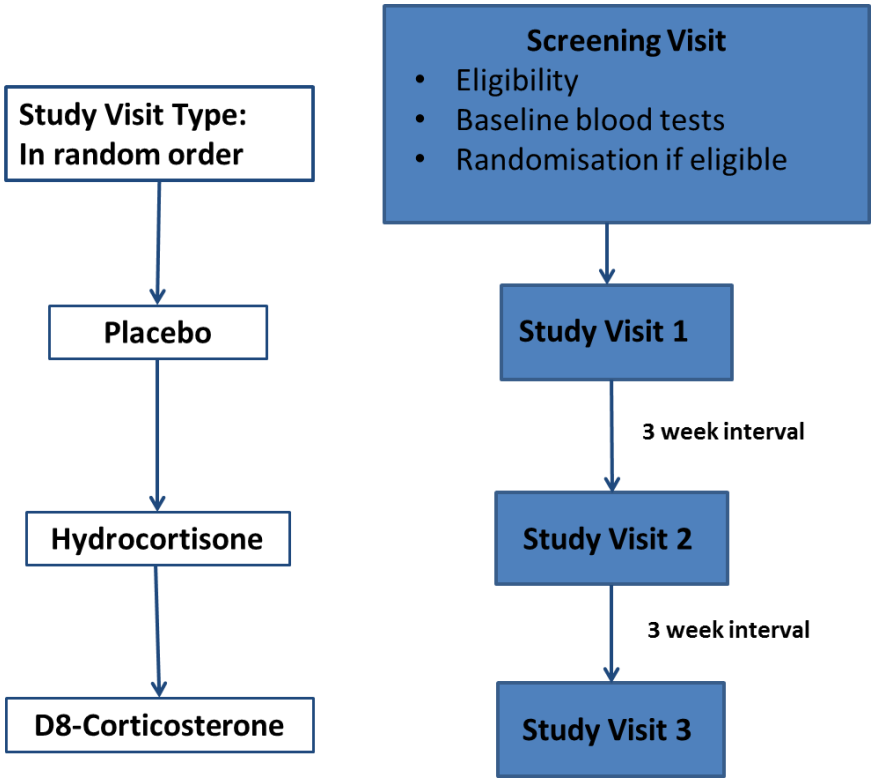
Data from a study involving 10 patients with Addison's disease were used to calculate sample size for this study (Nixon et al. 2016). In that study, patients were given either D8-corticosterone or 9,11,12,12-[^{2}H]$_{4}$-cortisol (D4-cortisol) and ACTH levels were measured at frequent intervals. Adipose tissue biopsies were obtained to look at downstream glucocorticoid signalling. Using these results, 16 patients provides greater than 85% power with a significance value of 0.05 to detect a difference of 15% in suppression of ACTH (this was observed in the pilot study) and over 90% power to detect a 30% increase in adipose tissue PER1 expression (this was increased by 60% in the previous work).

5.2.5 Clinical protocol

5.2.5.1 Study visits

Participants attended for three study visits and received placebo, cortisol and D8-corticosterone infusions in random order at least three weeks apart to allow washout between treatments, resolution of abdominal bruising and recovery of blood loss (Figure 5-1). Randomisation was undertaken by an independent researcher at the University of Edinburgh and both patient and study researcher were blinded until analysis was complete.

Figure 5-1: Study design

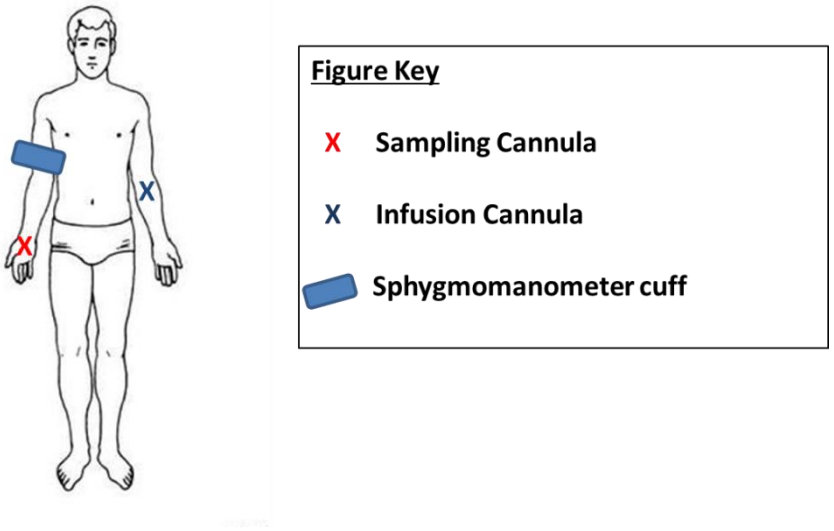


Participants attended the RIECRF at 8am having fasted from 10pm the night before. On the previous evening, usual evening glucocorticoid treatment was taken before 6pm. Normal morning glucocorticoid medication was omitted but all other medication including fludrocortisone was continued as usual. On arrival, participants had height, weight and blood pressure measured. Lean body mass and fat mass using bioelectrical impedance analysis was also measured as described in section 3.4.2.4.

Each participant was cannulated using an aseptic technique at two sites. A 20G cannula (Braun, Sheffield, UK) was inserted in the antecubital fossa of one arm for infusion of placebo/cortisol/corticosterone and tracers and a further retrograde 20G cannula (Braun, Sheffield, UK) was inserted in the dorsum of the contralateral hand for

sampling (Figure 5-2). The hand was placed in a hot box (manufactured in-house) heated to 55-60°C for 5 minutes prior to sampling in order to obtain arterialised samples. The sampling cannula was kept patent with a slow infusion of 0.9% Saline. Blood samples were taken from the retrograde cannula to measure glucocorticoids, androgens, 17-hydroxyprogesterone, ACTH, endogenous and tracer glucose and glycerol, insulin, non-esterified fatty acids and bone markers.

Figure 5-2: Anatomical placement of cannulas and sphygmomanometer cuff



At t-30 minutes, D2-glucose and D5-glycerol were given as a bolus of 1.66 μmol/kg/min and 17.6 μmol/kg/min respectively over 5 minutes followed by a constant infusion at 0.22 μmol/kg/min and 0.11 μmol/kg/min respectively for 355 minutes (Figure 5-3). At t=0 minutes, either placebo, hydrocortisone (0.94 mg) or D8-corticosterone (2.55 mg) was given as a bolus over 5 minutes then an infusion (hydrocortisone 2.71 mg at 44.8 ml/hr and D8-corticosterone 17.3 mg at 47.1 ml/hr) to achieve a concentration of 400nM for 145 minutes. A further identical bolus was

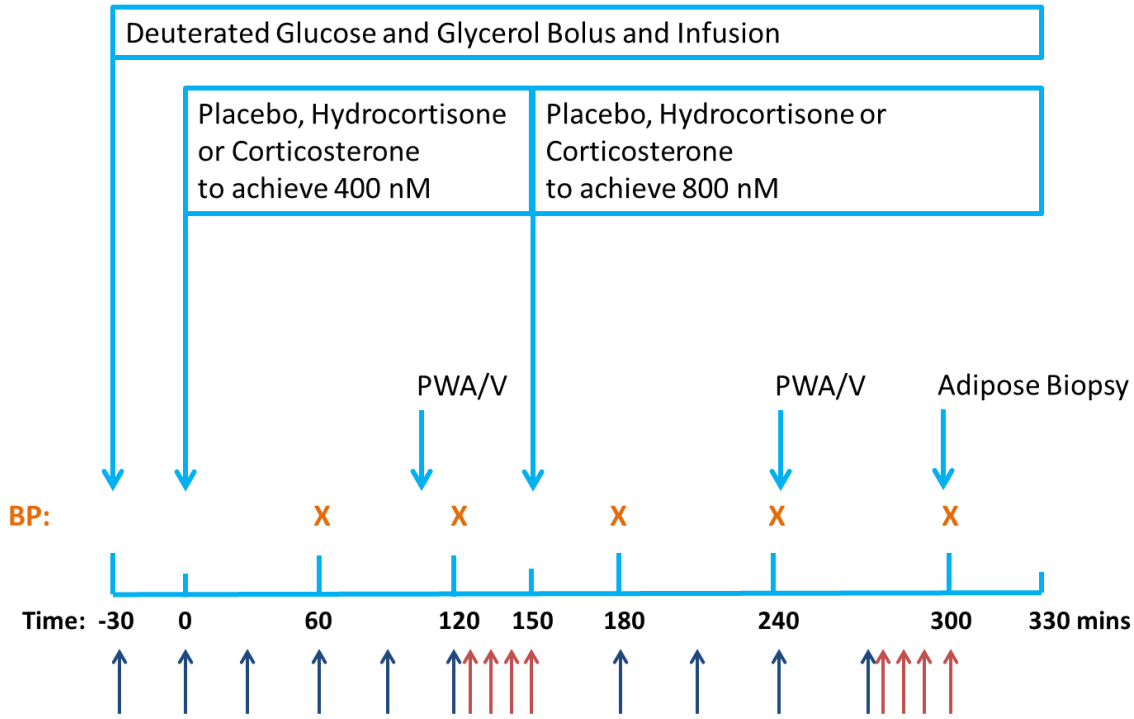
given at t+150 minutes followed by an infusion (hydrocortisone 6.53 mg at 89.6 ml/hr and D8-corticosterone 41.77 mg at 94.2 ml/hr) to achieve a steady state concentration of 800nM until the end of the study. Blood sampling was every 30 minutes from t-30 minutes until t+120 minutes when the frequency increased to every 7.5 minutes until t+150 minutes. Sampling returned to 30 minute intervals thereafter. At t+270 minutes, 7.5 minute sampling resumed until the final sample at t+300 minutes. Blood pressure was measured hourly using an average of two readings. Pulse wave analysis and velocity were measured at t+100 minutes and t+250 minutes. An abdominal fat biopsy was taken at t+300 minutes as described in section 4.2.5.5. At t+330 minutes, the infusions were discontinued, all cannulae removed, participants were given lunch and allowed home.

5.2.5.2 Sample collection and processing

Samples were obtained in plasma lithium heparin (7.5 mL), serum gel (4.9 or 7.5 mL) and potassium EDTA tubes (2.7 or 4.9 mL, all Monovette®, Starstedt, Numbrecht, Germany). Samples were gently mixed by inverting several times. All samples were subject to centrifugation immediately after sampling, separated and stored at -80°C for analysis. Lithium heparin and serum samples were subject to centrifugation at 2000 g for 10 minutes at 4 °C and EDTA samples at 2500 g for 10 minutes at 4 °C.

Figure 5-3: Study diagram

Blood pressure (BP) was measured hourly at time points indicated with X. Pulse wave analysis (PWA) and pulse wave velocity (PWV) were measured at the end of the low and high glucocorticoid concentration period. Blood samples were taken at 30 minute intervals (blue arrows) and at 7.5 minute intervals (red arrows) at the end of the low and high glucocorticoid concentration period.



5.2.5.3 Preparation of stable isotope tracers

D2-glucose and D5-glycerol (Euroisotop, Saint-Aubin, France) were prepared in water for injection, filtered by Tayside Pharmaceuticals to produce sterile stock solution and stored at -20°C for maximum 12 months. Solutions were prepared in the RIECRF on the morning of each study visit.

D8-corticosterone (Cambridge Isotope Laboratories, Andover, MA) was prepared in pharmaceutical grade ethanol/water (90/10%) and filtered by Tayside Pharmaceuticals

to produce sterile stock solution. This was stored at -20°C for maximum 12 months. Solutions were prepared in the RIECRF on the morning of each study visit.

5.2.5.4 D2-glucose and D5-glycerol infusion protocol

The deuterated glucose and glycerol infusions were infused on a weight dependent basis. If the participant was under 73 kg, the infusion was made up in 500 mL sodium chloride 0.9% *w/v* while those who weighed over 73 kg had infusions made up in 1000 mL sodium chloride 0.9% *w/v*.

D5-glycerol stock solution contained 330 mg in 33 mL (10mg/mL) of water for injection. D2-glucose stock solution contained 1.76 g in 22 mL (80mg/mL) of water for injection.

The bolus dose was prepared with 2 mL of 10 mg/mL D5-glycerol stock solution added to 13 mL sodium chloride 0.9% *w/v* followed by 5 mL of 80 mg/mL D2-glucose stock solution. A final concentration of 1 mg/mL of D5-glycerol and 20 mg/mL of D2-glucose was achieved. An intravenous bolus of 17.6 μmol/kg/min and 1.66 μmol/kg/min for glycerol and glucose respectively was given over 5 minutes.

The 500 mL infusion was prepared with 30 mL of 10 mg/mL D5-glycerol and 14 mL of 80 mg/mL D2-glucose added to 456 mL of sodium chloride 0.9% *w/v* to give a final concentration of 0.6 and 2.24 mg/mL respectively.

The 1000 mL infusion was prepared with 60 mL of 10 mg/mL D5-glycerol and 28 mL of 80 mg/mL D2-glucose added to 912 mL of sodium chloride 0.9% *w/v* to give the same final concentration of 0.6 and 2.24 mg/mL respectively.

5.2.5.5 D8-corticosterone infusion protocol

D8-corticosterone stock solution (Cambridge Isotopes, Andover, MA) contained 83.6 mg in 5.5 mL (15.2mg/mL) of ethanol/water (90/10%).

The infusion was prepared with 5 mL of 15.2 mg/mL stock solution added to 495 mL sodium chloride 0.9% *w/v* to give a final concentration of 0.152 mg/mL. An intravenous bolus of 2.55 mg was given over 5 minutes at 30.6 mg/hr followed by 17.3 mg over 145 minutes at 7.16 mg/hr. A further bolus of 2.55 mg over 5 minutes was given at 150 minutes followed by 41.77 mg over 175 minutes at 14.32 mg/hr.

5.2.5.6 Hydrocortisone infusion protocol

Hydrocortisone sodium phosphate (Concordia, Ontario, Canada) contained 100 mg in 1 mL of sterile aqueous solution (100 mg/mL).

The infusion was prepared with 1 mL of 100 mg/mL hydrocortisone added to 19 mL water for injection to give a final concentration of 5 mg/mL. 2.5 mL of this solution was added to 497.5 mL sodium chloride 0.9% *w/v* to give a final concentration of 0.025 mg/mL. An intravenous bolus of 0.94 mg was given over 5 minutes at a rate of 11.28 mg/hr followed by 2.71 mg over 145 minutes at 1.12 mg/hr. A further bolus of 0.94 mg over 5 minutes was given at 150 minutes followed by 6.53 mg over 175 minutes at 2.24 mg/hr.

5.2.5.7 Placebo infusion protocol

A 500 mL bag of sodium chloride 0.9% w/v (Baxter, Newbury, UK) was infused at 200 mL/hr for 5 minutes then 45 mL/hr for 145 minutes. At 150 minutes, a further

bolus of 200 mL/hr over 5 minutes was given and the infusion continued at 90 mL/hr until the end of the study.

5.2.5.8 Pulse wave analysis

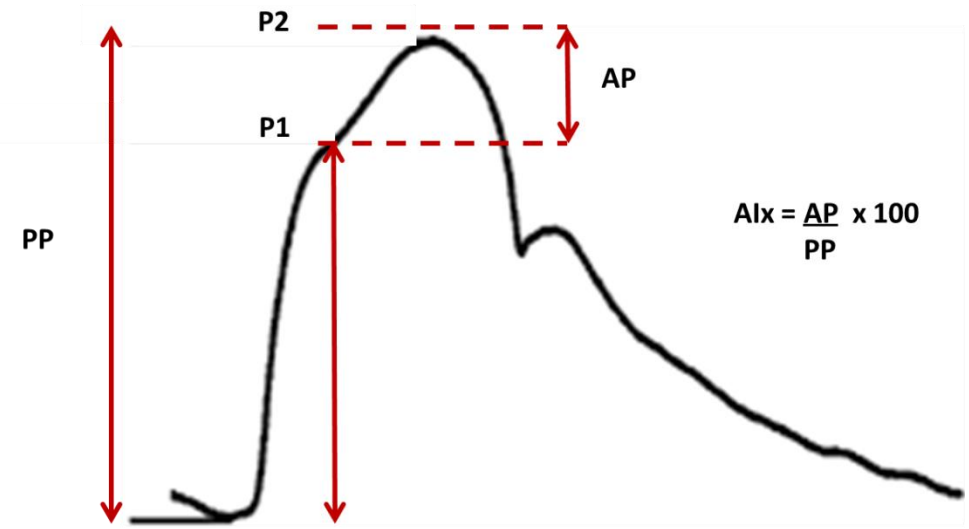
Pulse wave analysis (PWA) was performed at the radial artery by applanation tonometry using a SphygmoCor device (AtCor Medical Inc, Illinois, USA). The radial pulse waveform was recorded and central aortic pressure was derived using an automated generalised transfer function (O'Rourke & Gallagher 1996). The augmentation index was calculated as the increment in pressure from the shoulder of the ascending pressure wave to the peak of the reflected wave (Figure 5-4). To correct for the effect of pulse rate, augmentation index results were normalised for a heart rate of 75 bpm.

5.2.5.9 Pulse wave velocity

Carotid-femoral pulse wave velocity (PWV) was quantified using the SphygmoCor device (AtCor Medical Inc, Illinois, USA). The distance between the carotid and femoral arterial capture sites and the sternal notch were measured. Applanation tonometry was performed at each site and the arterial waveform was recorded. Simultaneous electrocardiogram monitoring was performed and the R-wave was used as a reference point for PWV calculations. PWV was calculated from the difference in transit time of the pulse wave to the carotid and femoral arteries.

Figure 5-4: Aortic pressure wave

The aortic pressure wave is derived from the radial pulse wave using a validated algorithm and is the sum of all forward and backward waves. P1 represents the first wave and P2 is the reflected second wave. The augmentation pressure (AP) is the contribution of the reflected wave to pulse pressure (PP). Augmentation index (AIx) represents the relationship between augmentation pressure and pulse pressure.



5.2.5.10 Biopsy of subcutaneous abdominal fat

A biopsy of subcutaneous fat was undertaken at t+300 minutes. This was carried out as described in section 4.2.5.5. Up to three aliquots were taken.

5.2.6 Sample analysis

5.2.6.1 Analytes

In addition to the labelled and unlabelled steroids in section 2.3.1.2, testosterone, androstenedione, 17-OH progesterone and D8-corticosterone were analysed. The above standards were supplied by Cambridge Isotopes.

5.2.6.2 Preparation of standard curves

Standard curves were prepared for all analytes across a range of 0.01-100 ng with D4-cortisol and epi-corticosterone added as internal standards (10 ng) (Figure 5-1). 1% bovine serum albumin (BSA) was used instead of water as a diluent and 0.5M ammonium hydroxide was used to make up each standard to 400 μL.

Table 5-1: Standard curve analytes and internal standards

Analyte	Standard Curve Range (ng)	Internal Standard	Internal Standard amount (ng)
Cortisol	0.01-100	D4-cortisol	10
Cortisone	0.01-100	D4-cortisol	10
Corticosterone	0.01-100	Epi-corticosterone	10
11-dehydrocorticosterone	0.01-100	Epi-corticosterone	10
17-OHP	0.01-100	D4-cortisol	10
Testosterone	0.01-100	D4-cortisol	10
Androstenedione	0.01-100	D4-cortisol	10

5.2.6.3 Extraction of plasma samples

Plasma samples were extracted as described in section 2.3.3 using 0.5M ammonium hydroxide instead of water to make up the solution. Internal standards D4-cortisol and epi-corticosterone (10 ng) were added to each sample.

5.2.6.4 LC-MS/MS analysis

Samples were analysed using the glucocorticoid and androgen assay as described in section 2.3.6.3.

5.2.7 Data analysis

LC-MS/MS data were analysed as described in section 2.3.7. Clearance of infused glucocorticoids cortisol and D8-corticosterone were calculated using Equation 5-1. The first period of steady state was t+30-150 minutes and the second period was t+180-300 minutes.

Equation 5-1

$$Clearance\ (L/\text{min}) = \frac{Rate\ of\ Infusion\ (nmol/min)}{Steady\ State\ concentration\ (nmol/L)}$$

In order to compare glucocorticoid effect on disease control, percentage change in ACTH from baseline was calculated and plotted against detected glucocorticoid concentration. Linear regression was performed for each participant and validity was confirmed by comparison against zero. For each participant, the dose of glucocorticoid required to suppress ACTH by 50% was calculated and mean ± SEM for cortisol and D8-corticosterone is presented. Data were analysed using GraphPad Prism® Version 6.01.

5.3 Results

We plan to recruit 16 participants to this study and both recruitment and study visits continue. The data presented in this chapter are for the 8 participants who have completed all three study visits to date. Analysis of D2-glucose and D5-glycerol is incomplete and has been omitted. The LC-MS/MS method included testosterone however this data was not robust and requires further analysis. We will complete plasma analysis with bone markers of resorption and formation, CTX and P1NP respectively. Adipose biopsy samples have not yet been analysed.

5.3.1 Characteristics of study participants

Characteristics of participants are presented in Table 5-2. Participants were taking a variety of glucocorticoid therapies at different doses and times of the day: hydrocortisone (37.5%), prednisolone (50%) or a combination (12.5%). Concomitant treatment with fludrocortisone was taken by 87.5% of participants.

Table 5-2: Participant demographic data

Data are for n=8 (6 female). Daily equivalent hydrocortisone dose calculated using British National Formulary conversion chart where prednisolone 5mg = hydrocortisone 20mg.

	Mean ± standard deviation	**Range**
Age (years)	39.1±13.0	25-59
Height (m)	1.6±0.1	1.4-1.7
BMI (kg/m^2)	31.2±12.8	20.6-52.7
Bioimpedance:		
% Fat	30.5±7.8	18.2-42.9
Mass (kg)	21.9±10.8	11.3-44.6
Waist: Hip Ratio	1.0±0.1	0.8-1.0
Daily equivalent hydrocortisone dose (mg)	19.1±4.0	15-25
Daily fludrocortisone dose (µg)	175.0±140.5	50-400

5.3.2 Plasma glucocorticoids

Measurement of cortisol and corticosterone concentrations during the infusions indicated ramped steady state had been achieved (Figure 5-5). Baseline cortisol concentrations were low (mean ± SEM 35.71 ± 12.12 nmol/L) and D8-corticosterone was undetectable. Steady state concentrations of corticosterone were similar to those intended, however cortisol concentrations were substantially lower than planned.

Clearance of cortisol and D8-corticosterone were calculated using Equation 5-1 and are shown in Table 5-3.

Figure 5-5: Plasma glucocorticoid concentrations

Data are mean ± SEM for plasma cortisol (blue line) and corticosterone (red line) concentrations at time points -30-300 minutes (both n=8). Intended circulating concentration indicated with black dashed line.

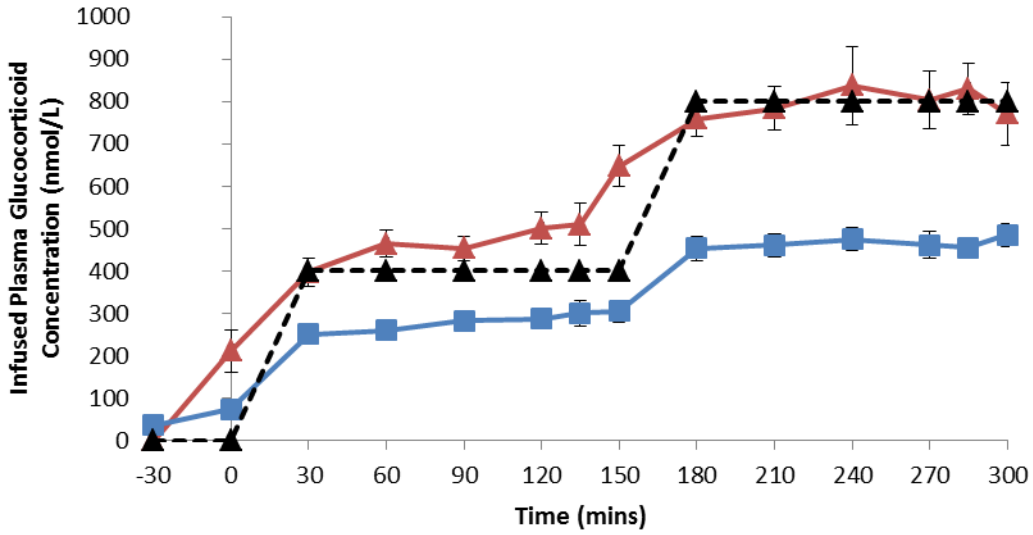


Table 5-3: Plasma glucocorticoid clearance

Data are mean ± SEM for n=8.

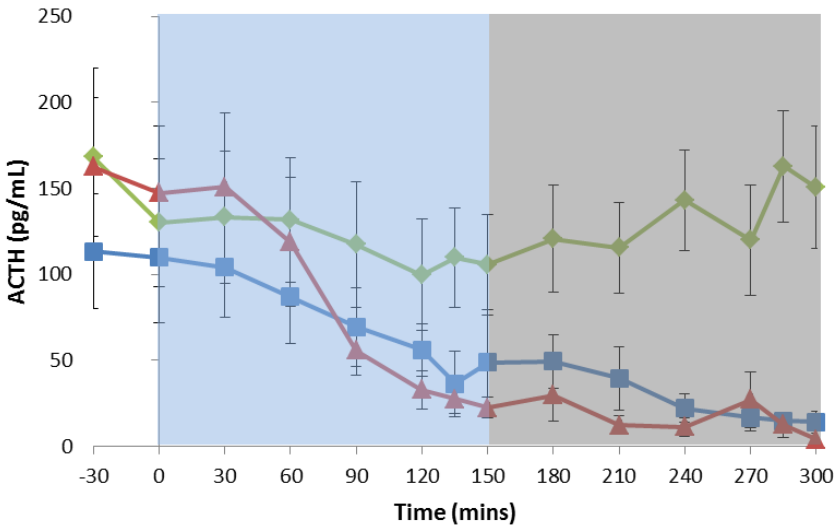
Time (mins)	Cortisol Clearance (L/min)	D8-corticosterone Clearance (L/min)
30-150	0.19±0.01	0.72±0.04
180-300	0.23±0.01	0.92±0.06

5.3.3 Plasma ACTH concentration

Plasma ACTH for each phase is shown in Figure 5-6. ACTH at baseline was elevated above the normal range in all phases and fell with time during both cortisol and D8-corticosterone phases. There appeared to be no clear change with time during the placebo phase.

Figure 5-6: Plasma ACTH

Data are mean ± SEM for plasma ACTH for placebo (green line), cortisol (blue line) and D8-corticosterone (red line) phases at time points -30-300 minutes (all n=8). The blue shaded area represents the first steady state glucocorticoid dose and the grey area is the second higher dose.



The percentage change in ACTH from baseline was calculated and plotted against achieved plasma concentration of glucocorticoid (Figure 5-7). Linear regression was applied to each individual plot and the glucocorticoid concentration at which 50% suppression of ACTH was achieved is summarised in Table 5-4.

Figure 5-7: Linear regression of % suppression ACTH from baseline and plasma glucocorticoid concentration.

Data are for n=7 as subject 8 was an outlier.

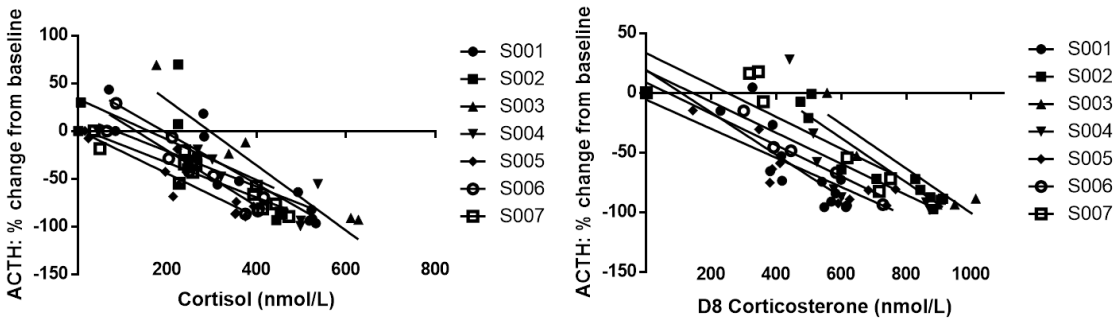


Table 5-4: Linear regression of percentage suppression ACTH from baseline and plasma glucocorticoid concentration.

Slope of linear regression for cortisol (F) and D8-corticosterone (D8B) for each participant were calculated and goodness of fit is shown with R^2 value. Those slopes starred (*) were significantly different from zero. 50% ACTH suppression was calculated from slope equations using y= -50. This was calculated only for slopes significantly different to zero.

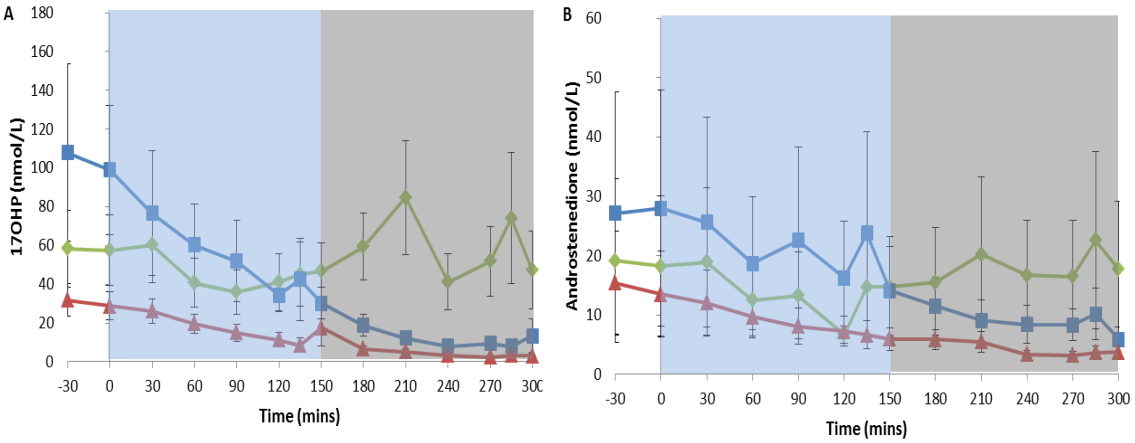
Subject	Linear Fit					
	Slope		R^2		50% ACTH suppression (nmol/L)	
	F	D8B	F	D8B	F	D8B
S001	Y=-0.2662x+51.23	Y=-0.1772x+19.02	0.8355*	0.7546*	380.3	389.5
S002	Y=-0.2127x+33.86	Y=-0.1850x+73.14	0.3914	0.8754*	-	665.6
S003	Y=-0.3413+100.7	Y=-0.1868x+86.1	0.7939*	0.8461*	441.5	728.6
S004	Y=-0.1850x+15.89	Y=-0.1298x+18.5	0.7552*	0.5766*	356.2	527.7
S005	Y=-0.2274x+1.123	Y=-0.1217x-5.786	0.8474*	0.838*	224.8	363.3
S006	Y=-0.2915x+38.14	Y=-0.1311x+8.648	0.8937*	0.9353*	302.4	447.4
S007	Y=-0.1825x+3.531	Y=-0.1333x+33.21	0.8691*	0.7052*	293.3	624.2
S008	Y=-0.2555x+127.5	Y=0.00075x+0.58	0.1533	0.0165	-	-
Mean ± SEM	-	-	-	-	**333.1 ± 31.0**	**535.2 ± 53.6**

5.3.4 Plasma 17-OHP and androstenedione concentration

Plasma 17-OHP and androstenedione were measured in arterialised plasma samples for each phase and are shown in Figure 5-8. Both were elevated above the normal range at baseline. 17-OHP appeared to be suppressed in both cortisol and D8-corticosterone phases with time and remained elevated in placebo phase. Androstenedione concentrations followed a similar pattern.

Figure 5-8: Plasma 17-OHP and androstenedione

Data are mean ± SEM for A) plasma 17-OH progesterone (17OHP) and B) androstenedione in arterialised samples at time points -30-300 minutes during placebo (green line), cortisol (blue line) and D8-corticosterone (red line) phases (all n=8). The blue shaded area represents the first steady state glucocorticoid dose and the grey area is the second higher dose.



5.3.5 Metabolic markers of glucocorticoid action

5.3.5.1 Blood pressure

Blood pressure was measured at regular intervals during the study visit and are presented in Figure 5-9. Both systolic and diastolic blood pressure appeared similar in each phase.

5.3.5.2 Pulse wave assessment

Both pulse wave analysis and velocity appeared unchanged over the study period and in each phase (Figure 5-10).

Figure 5-9: Blood pressure

Data are mean ± SEM for systolic (unbroken line) and diastolic (dashed line) blood pressure (mmHg) for placebo (green lines), cortisol (blue lines) and D8-corticosterone (red lines) (n=8). The blue shaded area represents the first steady state glucocorticoid dose and the grey area is the second higher dose.

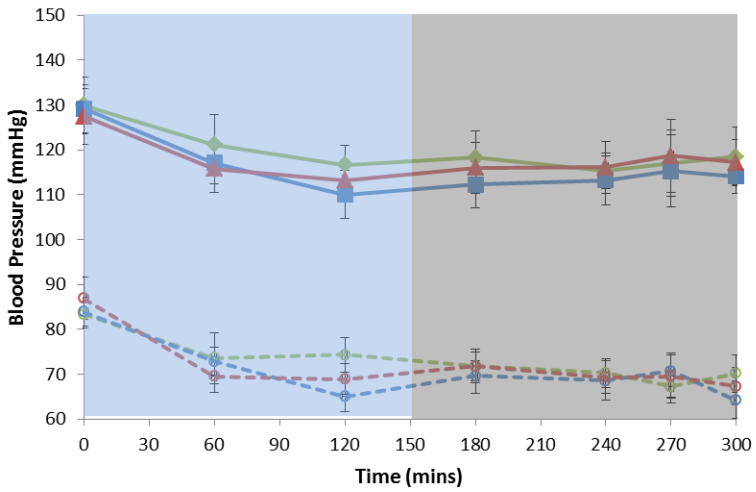
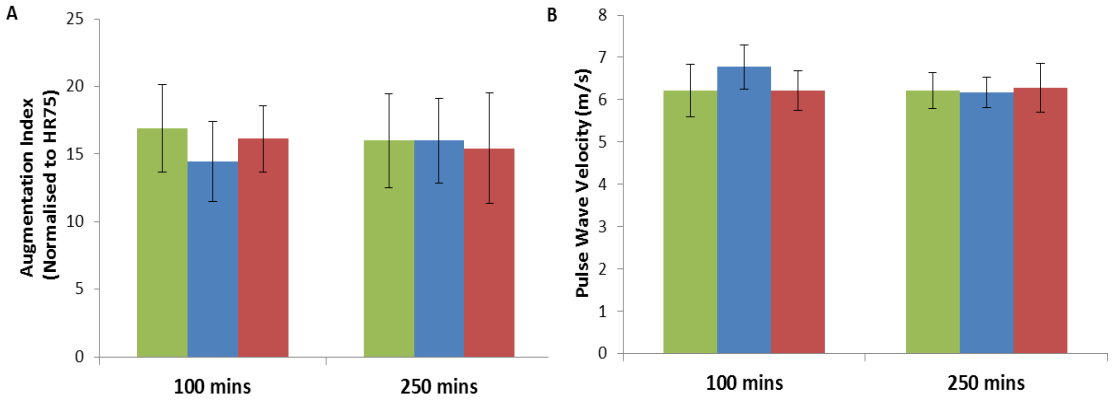


Figure 5-10: Pulse wave analysis and velocity

Data are mean ± SEM for A) pulse wave analysis and B) pulse wave velocity during placebo (green fill), cortisol (blue fill) and D8-corticosterone (red fill) phases (all n=8). Pulse wave analysis was measured using augmentation index (normalised to HR 75) and pulse wave velocity using metres per second (m/s).



5.3.5.3 Insulin

Plasma insulin was measured at intervals throughout the study period (Figure 5-11). There was a suggestion that the corticosterone phase is associated with lower circulating insulin compared to placebo and cortisol phases.

5.3.5.4 Non-esterified fatty acids

Non-esterified fatty acids (NEFAs) were measured regularly throughout the study period (Figure 5-12). NEFAs appeared to be similar in all study phases.

Figure 5-11: Plasma insulin

Data are mean ± SEM for plasma insulin during placebo (green line), cortisol (blue line) and D8-corticosterone (red line) phases at time points -30-300 minutes (n=8). The blue shaded area represents the first steady state glucocorticoid dose and the grey area is the second higher dose.

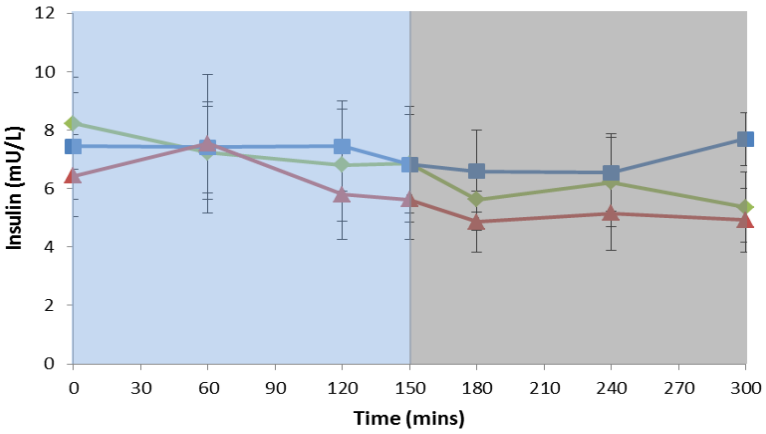
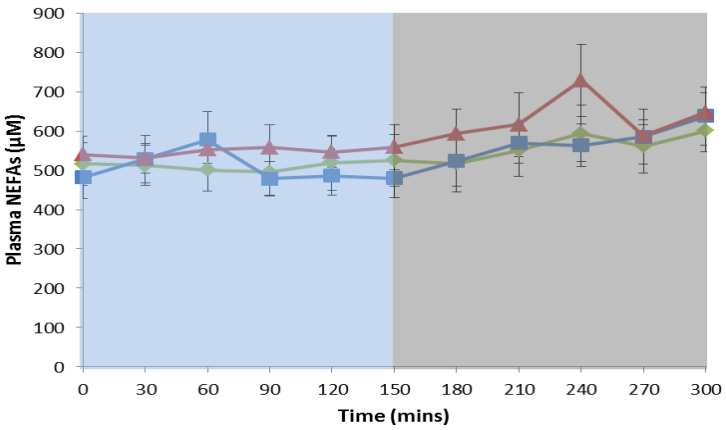


Figure 5-12: Plasma NEFAs

Data are mean ± SEM for plasma NEFAs during placebo (green line), cortisol (blue line) and D8-corticosterone (red line) phases at time points -30-300 minutes (n=8). The blue shaded area represents the first steady state glucocorticoid dose and the grey area is the second higher dose.



5.4 Discussion

This randomised placebo-controlled crossover study comparing the biochemical and metabolic effects of cortisol and corticosterone in CAH is ongoing and the data we present represents the midway point. We have chosen not to perform an interim statistical analysis as we are powered for 16 participants and as such the results would likely be misleading. Therefore, the data is presented without statistical analysis.

We chose to study patients with CAH as they are the group who could benefit most from corticosterone therapy. Outcomes in patients with CAH are poor with current therapy and we hypothesised that corticosterone may provide adequate glucocorticoid replacement and adrenal androgen suppression with fewer metabolic side effects. In comparison with the UK cohort studied as part of the Congenital Adrenal Hyperplasia Adult Study Executive (CaHASE), our subjects are also on a variety of glucocorticoid replacement regimens, with prednisolone the most common. We had no patients taking dexamethasone in contrast to 24% of males and 17% of females in CaHASE (Arlt et al. 2010). This may reflect changing prescribing tendencies as more evidence suggests dexamethasone is associated with greater insulin resistance and poor metabolic outcomes (Han, Stimson, et al. 2013). Most of our patients also required mineralocorticoid replacement with 87.5% on fludrocortisone, compared to 72-82% of the CaHASE cohort. In addition, our patient group are similarly short in height with raised BMI in line with the UK cohort.

It is striking from the analysis of infused glucocorticoid concentration that our intention of matching cortisol and corticosterone plasma concentrations was

unsuccessful. Cortisol reached a steady state of approximately 250 nmol/L initially and only rose to approximately 400 nmol/L during the second steady state period. In contrast, plasma D8-corticosterone concentrations were much closer to our intended steady state concentrations of 400 nmol/L and 800 nmol/L. The reasons for this discrepancy are unclear.

We considered the possibility that this result may have been due to analytical error during the LC-MS/MS analysis. The analysis was revisited and repeated using freshly prepared standard curves and a selection of random samples. This confirmed the original results.

A further possibility was that incorrect assumptions were made in calculating the rate of cortisol infusion. This calculation was based on a previous *in vivo* study in patients with Addison's disease (Nixon et al. 2016). In this study, D4-cortisol rather than hydrocortisone was infused to achieve three ramped steady state concentrations at 25, 100 and 250 nmol/L. ACTH was only marginally suppressed at the end of the study period and these concentrations were relatively low when compared to physiological concentrations. Although D4-cortisol and hydrocortisone are similar, they are not interchangeable when considering pharmacokinetics as clearance of D4-cortisol is more rapid when compared to cortisol (0.46 vs 0.28 L/min respectively) (Andrew et al. 2002). This is due to regeneration of D3-cortisol rather than D4-cortisol from D3-cortisone unlike the reversible interconversion of cortisol and cortisone via 11β-HSD. With this in mind, it might be expected that we would have given more hydrocortisone then necessary and achieved greater than planned steady state concentrations however

the opposite is true. It is not clear why this is the case and may suggest an alternative explanation for these findings.

A final consideration is that patients with CAH have altered glucocorticoid metabolism. These patients have genetically disrupted steroidogenesis and it is possible that they handle glucocorticoids differently to healthy individuals. While cortisol pharmacokinetics have not been extensively investigated in CAH patients, there is considerable recent data comparing conventional and modified release preparations of hydrocortisone (Newell-Price et al. 2008; Verma et al. 2010; Mallappa et al. 2015). This is summarised by Mallappa et al and shows similar area under the curve concentrations over 24 hours for modified release hydrocortisone in CAH and healthy controls. There is limited literature directly measuring clearance in this group of patients but Charmandari et al have investigated cortisol pharmacokinetics in pre-pubertal, pubertal and post-pubertal patients with CAH. In this study, cortisol clearance in pre- and post-pubertal participants (0.25 and 0.29 L/min) were similar to published values in healthy individuals. During puberty, there was an observed increase in cortisol clearance (0.43 L/min) which was hypothesised to be secondary to the other endocrine changes occurring during this period (Charmandari et al. 2001). The clearance of cortisol in healthy individuals is estimated at between 0.28 and 0.33 L/min (Andrew et al. 2002; Stimson et al. 2007) and this is similar to that calculated by Charmandari et al in the post-pubescent phase. The calculated clearance of cortisol in our study is marginally reduced at 0.19-0.23 L/min and if anything, one might expect accumulation of cortisol. It seems likely therefore that we have under-estimated the hydrocortisone dose required to achieve our target plasma concentrations.

While the achieved glucocorticoid concentrations differ during each steady state period, a comparison can be made of the plasma concentration of cortisol and corticosterone required to suppress biochemical markers of CAH by 50%. This could be analysed with either ACTH or adrenal androgens however ACTH was used as a more direct marker of suppression of the HPA axis. Using linear regression, glucocorticoid concentration was plotted against percentage suppression of ACTH. One participant had very low concentration of ACTH from baseline and throughout each study visit. This may reflect a prolonged effect of glucocorticoid treatment despite taking their evening dose prior to 6pm the night before or simply non-compliance with study instructions. As such, glucocorticoid concentrations for 50% reduction in ACTH in this participant could not be calculated. One further participant was an outlier in the cortisol phase and had a slower response with ACTH initially rising before falling. This meant the linear regression was not significant and could not be analysed.

From the data available, the concentration of corticosterone required to suppress ACTH by 50% is higher than cortisol which may suggest corticosterone is less potent than cortisol. It is difficult to make any clear conclusions at this stage however as this represents only half the intended data. While this initial data suggests corticosterone may be less potent than cortisol, it does show corticosterone has a suppressive effect on the HPA axis which has not previously been demonstrated in CAH patients. This is supported by the adrenal androgen data where both glucocorticoid phases suppress 17-OHP and androstenedione to normal values by the end of the study period.

At this stage, there does not appear to be any clear difference in metabolic markers between study visits although any difference is likely to be small and may not yet be apparent. There are some limitations in assessing this response, in particular, the time of exposure to glucocorticoid and the likelihood of seeing a difference in effect. The difference in our achieved glucocorticoid concentrations will also complicate analysis. While we can compare the dose achieved in the first phase of D8-corticosterone and the second phase of cortisol, there will also be a significant impact of time which will not be comparable. The placebo phase will be useful in controlling for the normal diurnal variation of these markers.

The mechanism of glucocorticoid effect on blood pressure is likely to be multifactorial mediated by renal sodium retention, plasma volume expansion and increased peripheral resistance (Walker 2007). Most studies assessing impact of glucocorticoids on blood pressure look at outcomes over a much longer period of time with follow up assessed after months rather than hours (Mallappa et al. 2015; Giordano et al. 2016). It is therefore possible that we will not see any significant difference in blood pressure in this short 5.5 hour study period.

Non-invasive measures of cardiovascular parameters are useful in assessing the mechanism of increased cardiovascular risk. PWV is a gold standard measure of arterial stiffness and is an independent predictor of cardiovascular events. A meta-analysis of studies which evaluated PWV found an increase by 1 m/s was associated with 15% increase in total cardiovascular events (Vlachopolous et al. 2010). Similarly, PWA measured by the augmentation index is a measure of left ventricular systolic

loading and a surrogate marker of arterial stiffness. Another meta-analysis by the same authors demonstrated that a 10% increase in augmentation index was associated with a 30% increased risk of cardiovascular events (Vlachopoulos et al. 2010). Our results show no obvious difference between groups at present but this is a sensitive marker of cardiovascular risk and comparing the percentage change with glucocorticoid dose would be a clinically useful evaluation. A recent study investigated the effect of a short term (7 days) increase in glucocorticoid replacement dose on insulin sensitivity with change in pulse wave analysis as the primary outcome (Petersons et al. 2014). They reported no change in augmentation index which perhaps highlights the need for longer term studies before an effect will be observed.

In the previous comparison of cortisol and corticosterone in Addison's disease, circulating metabolic markers, insulin and NEFAs were unchanged during the study period. Our study benefits from the addition of a placebo phase to show baseline effects on these markers without circulating glucocorticoids.

There is a trend for insulin to fall over the study period when exposed to corticosterone in comparison to cortisol. The placebo phase appears to fall with time too but to a lesser extent. Considering the corticosterone plasma exposure is almost double that of cortisol, this would be a significant outcome should the final results be similar.

NEFAs appear to increase gradually over the study period but there is no clear divergence from either glucocorticoid phase. Previous studies have shown differing effects of glucocorticoids on lipolysis and insulin secretion with most effect seen after several hours of supra-physiological glucocorticoid exposure (Dinneen & et al. 1993;

Djurhuus et al. 2002). A recent study has shown short term exposure to cortisol at similar concentration to those achieved in this study had no effect on lipolysis (Stimson et al. 2017).

In summary, our interim results demonstrate that corticosterone is a potential treatment for CAH with evidence it suppresses ACTH drive and subsequent adrenal androgen production. There does not appear to be any difference in metabolic markers between cortisol and corticosterone at this stage and it may be challenging to compare the metabolic effects with the difference in achieved glucocorticoid concentrations. Final analysis with a full data set will determine whether corticosterone is equipotent to cortisol and whether any difference in metabolic side effects can be detected. As discussed above, a number of the metabolic effects we are interested in preventing are best assessed after a longer term exposure to glucocorticoid therapy. The next step in our investigation of corticosterone as an alternative to cortisol would be to develop an oral formulation which could be taken over a period of months and compared to current treatment. This would allow longer term follow up of cardiovascular parameters.

Chapter 6. Conclusions

Despite being the first glucocorticoid identified by Hench, Kendall and Reichstein in 1937, the role of corticosterone in humans remains poorly defined. Circulating at relatively low plasma concentration, it has long been assumed to have little role over and above cortisol, the more abundant glucocorticoid in humans. Increasingly, evidence is emerging of distinct tissue-specific regulation of these glucocorticoids, particularly by the differential expression of ABC transporters ABCB1 and ABCC1 which selectively export cortisol and corticosterone respectively (Webster & Carlstedt-Duke 2002; Karssen et al. 2001; Raubenheimer et al. 2006). We propose an alternative HPA axis which accounts for the actions of both glucocorticoids in different tissues. The higher relative expression of ABCB1 at the blood brain barrier excluding cortisol but not corticosterone suggests corticosterone may have a more significant role in negative feedback of the HPA axis. Increased expression of ABCC1 in adipose tissue and skeletal muscle suggests cortisol is the principal glucocorticoid acting in these tissues with corticosterone actively exported. We hypothesise that corticosterone would therefore be a suitable alternative glucocorticoid replacement therapy in patients with Addison's disease and CAH. Doses of corticosterone required to suppress the HPA axis may be relatively lower than current standard treatment with hydrocortisone. The presence of ABCC1 in tissues such as adipose and skeletal muscle may protect these tissues from accumulation of corticosterone and subsequently lead to fewer metabolic side effects.

The studies presented in this thesis explore the differences between cortisol and corticosterone in different human biological samples and further characterise the role of ABCC1 in central negative feedback of the HPA axis and in corticosteroid receptor

occupancy of peripheral tissues. The first human data using corticosterone as a glucocorticoid replacement therapy in patients with CAH is also presented.

Our data highlights differences in relative cortisol and corticosterone concentrations within different biological samples. Measuring glucocorticoids in hair is increasingly being used as an effective measure of long term glucocorticoid exposure. Corticosterone had never, to our knowledge, been measured in hair and we anticipated this may be difficult due to the already low concentration of cortisol in previous reports (Raul et al. 2004; Manenschijn et al. 2011). Preliminary data from a single individual suggest corticosterone concentration is very low in hair and routine measurement is unlikely to be feasible due to the large volume of sample required. Further measurements in more subjects would be necessary to confirm these findings.

Our initial investigation of salivary corticosterone suggested a lack of diurnal rhythm in comparison to cortisol. In a clinical study obtaining paired plasma and saliva samples over a 14-hour period in healthy individuals, we were able to investigate the relationship between plasma and saliva corticosterone in humans for the first time using LC-MS/MS. Salivary corticosterone concentration is much lower than cortisol with no discernible circadian rhythm nor any correlation with plasma concentrations. Plasma corticosterone however has a similar circadian rhythm to cortisol and correlates well with salivary 11-dehydrocorticosterone which was present in higher concentration than corticosterone. Our results underline the role of 11β-HSD2 in the parotid gland with 11-dehydrocorticosterone a better measure of plasma corticosterone, similar to the relationship between salivary cortisone and plasma

cortisol (Perogamvros, Keevil, et al. 2010). We have demonstrated plasma corticosterone has a circadian rhythm similar to cortisol suggesting they are under similar regulation in the circulation. The differences in saliva and hair glucocorticoid concentrations may be secondary to differential local ABC transporter expression however this was not tested directly in this thesis.

Our proposed alternative HPA axis highlights the importance of ABC transporters in regulating tissue specific glucocorticoid concentration and we aimed to further define the role of ABCC1 in HPA axis negative feedback and peripheral corticosteroid receptor occupancy. Our hypothesis that ABCC1 inhibition would not impact negative feedback of the HPA axis was based on literature supporting the greater relative expression of ABCB1 over ABCC1 in the brain (Karssen et al. 2001; Raubenheimer et al. 2006). The higher relative concentration of corticosterone in brain and CSF suggests cortisol is exported via ABCB1 and central negative feedback is disproportionately influenced by corticosterone. Our data in fact shows that ABCC1 inhibition does influence HPA negative feedback with enhanced glucocorticoid secretion after combined corticosteroid receptor antagonism. This key finding suggests that ABCC1 is significant in negative feedback and given that ABCB1 expression is greater than ABCC1 at the blood brain barrier, this must be outside the brain itself. The pituitary gland lies out with the blood brain barrier and our study has highlighted the pituitary contribution to HPA negative feedback.

We did not demonstrate that corticosteroid receptor occupancy in peripheral tissues is influenced by ABCC1 inhibition. We used MR and GR antagonists potassium

canrenoate and mifepristone (RU486) to displace the bound glucocorticoids and measure release or uptake within different tissues using arterio-venous sampling. Displacement of cortisol from MR across the myocardium has been demonstrated before (Iqbal et al. 2014) however the increment in this study was small and it may be difficult to measure such small changes in tissue glucocorticoid concentrations. While we have not managed to prove our hypothesis, this may be due to lack of sensitivity of the technique. The data from *in vitro* and murine models remains compelling that ABCC1 exports corticosterone from these cells and may provide a mechanism by which we can protect metabolic tissues from excess corticosteroid exposure.

We present the first data in humans using corticosterone as glucocorticoid replacement therapy in patients with CAH. We hypothesised that corticosterone would provide a suitable glucocorticoid replacement therapy with potential for enhanced HPA axis negative feedback when compared to cortisol and that metabolic side effects would be minimised due to export of corticosterone from peripheral tissues such as adipose tissue and skeletal muscle. Our interim data shows that corticosterone is effective at suppressing the HPA axis with suppressed ACTH, 17-OHP and androstenedione. This has never been shown in patients with CAH before and is promising as an indication of the final results. The metabolic data shows no clear differences at present although the trend for lower circulating insulin in the corticosterone phase is interesting. As discussed previously, we may not see any significant changes in these metabolic markers over this short study period. We have yet to analyse the effects on glucose metabolism with *in vivo* deuterated glucose and glycerol measurements and the effect on bone markers P1NP and CTX. A final analysis of adipose biopsies to compare

glucocorticoid sensitive transcripts in each phase will give an indication of whether ABCC1 prevents accumulation of corticosterone and protects from over-activation of corticosteroid receptors.

Overall, one of the strengths of these studies is the use of LC-MS/MS to measure corticosterone which is a sensitive and specific method of quantification. The historical data available regarding corticosterone, while relatively extensive, is limited by the variety of assays used which have now largely been superseded by more modern techniques. Conducting *in vivo* studies was another strength, allowing assessment of corticosterone and ABC transporter activity in humans where before only *in vitro* and mouse models have been used. Both interventional clinical studies benefitted from a crossover design which allowed each participant to act as their own control, a particular strength when assessing individual hormone profiles. The inclusion of a placebo arm in the final study in CAH patients was particularly important to distinguish between normal diurnal variation of circulating hormones and true effects of cortisol and corticosterone.

As with many *in vivo* studies, these were not without their limitations. I have discussed already the difficulties in demonstrating displacement from individual tissues. This was further limited by the difficulties with abdominal vein cannulation which was a technically challenging procedure and restricted the analysis of adipose tissue displacement to half of the participating cohort. Consideration must always be made of off-target effects of study medication, particularly the use of probenecid, a drug with several therapeutic targets.

The findings of this thesis raise a number of important questions which ought to be addressed. Firstly, we have identified that corticosterone does not simply mimic cortisol in hair and saliva and is present in low concentration and lacks diurnal rhythm respectively. While we can speculate that these differences are related to differential ABC transporter expression, we have not directly tested this. Further investigation with ABCB1 and/or ABCC1 inhibition would clarify the influence these transporters have on glucocorticoid concentrations and add to our understanding of glucocorticoid metabolism in humans.

Our study of the physiological relevance and importance of ABC transporters in normal glucocorticoid metabolism has highlighted the role of ABCC1 in HPA negative feedback. We have inferred that this is through pituitary driven negative feedback given the evidence of ABCB1 action at the blood brain barrier and this raises further intriguing questions regarding the differential influence of cortisol and corticosterone at pituitary and hypothalamic sites. We can speculate that rapid induction of negative feedback is influenced primarily by cortisol at the pituitary and corticosterone at the hypothalamus and slower feedback via GR and MR will be influenced by relative binding affinities. This has implications in dissecting out ultradian regulation of the HPA axis and would warrant consideration of corticosterone as well as cortisol pulsatility in further studies.

In order to fully explore the relevance of both ABCB1 and ABCC1 in the regulation of the HPA axis, we would wish to examine the expression of ABC transporters in human pituitary and hypothalamus tissue samples in more detail. In addition, a further

parallel study assessing the effect of ABCB1 inhibition *in vivo* would be necessary. Using an ABCB1 inhibitor such as quinine, a similar displacement study may be undertaken with arteriovenous brain tissue sampling via jugular vein cannulation (Kilgour et al. 2015).

Subject to the final results of our study of corticosterone in CAH, the logical next step in assessing corticosterone as a replacement therapy would be to carry out a longer-term study. For this, we would require an oral formulation of corticosterone in a likely modified release form. A double-blind randomised cross over study design would be ideal and follow up ought to be at least 3 months for better assessment of metabolic outcomes.

Further work to determine the regulation of corticosterone in normal physiology will add to the mounting evidence that cortisol and corticosterone are under distinct tissue specific regulation. The potential metabolic benefits of using corticosterone as an alternative glucocorticoid replacement therapy are yet to be seen however we now have evidence that it can provide effective HPA axis suppression. The prospect of corticosterone as a new glucocorticoid replacement therapy joins a number of other new developments in this field including continuous subcutaneous hydrocortisone infusion, modified release hydrocortisone and inhibitors of *CYP17A1* (Nella et al. 2016; Mallappa et al. 2015; Auchus et al. 2014; Nilsson et al. 2017). This treatment is the only one with the prospect of reduced metabolic side effects while maintaining equal efficacy compared to hydrocortisone however and has real potential to make an impact on patient morbidity and mortality.

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