Improving Survival in Adrenal Insufficiency Disease-

Low-Dose Prednisolone versus Hydrocortisone

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Abstract

Glucocorticoid replacement therapy is essential to life in adrenal insufficiency (AI). Individuals with the condition have a life expectancy that is reduced by up to 12 years because of cardiometabolic adverse effects possibly due to mild over-replacement.

Hydrocortisone is the most common treatment given thrice daily and usually prescribed in a one-size-fits-all manner. Prednisolone is an alternative medication. Only recently has it been used at very low doses of 2-5 mg once daily, with doses tailored to individuals. There is a vacuum of evidence comparing its safety and efficacy to hydrocortisone, leading to hesitance in its uptake.

The Objective Markers and New Indicators in Adrenal Insufficiency Disease (OMNI-AID) study is a cross-sectional observational study that recruited healthy volunteers, AI patients receiving low-dose prednisolone, patients taking standard hydrocortisone and patients receiving high-dose glucocorticoids. Individuals attended a study visit, where anthropometric and subjective health data, blood and urine samples were collected. These were used to assess bone health, cardiovascular risk, glycaemic handling, immunity and subjective health, between the groups. The principal aim was to compare prednisolone and hydrocortisone therapy in the treatment of AI.

The results demonstrate no significant difference between cohorts of patients receiving hydrocortisone and prednisolone in any of the parameters measured. The cohort receiving hydrocortisone did have elevated markers of cardiovascular risk indicated by a higher high-sensitivity CRP and triglycerides than healthy volunteers. They were noted to have higher insulin levels and indices of insulin secretion, suggesting a hyperinsulinaemic normoglycaemic state. All glucocorticoid group patients showed elevated neutrophils compared to the healthy volunteers suggesting relative glucocorticoid over-exposure.

Taken together, this study presents data indicating minor differences between hydrocortisone and prednisolone replacement detectable only by comparison to healthy volunteers, with no overt difference between the two. This suggests that either medication can be safely used for the management of AI.

Copyright Declaration

Declaration of Originality

I declare that the text is of my own writing. Where appropriate I have cited other researchers' work. I completed the majority of the work described in this thesis. All collaboration and assistance has been detailed below.

Study visits were completed with the assistance of Dr Thilipan Thaventhiran, Dr Kleopatra Alexiadou and Dr Katharine Lazarus on occasions of my absence.

Chapter 3- Osteocalcin assays were performed with assistance of Dr Thilipan Thaventhiran.

Chapter 6- Flow cytometry protocol was designed with input from Dr Thilipan Thaventhiran. Flow cytometry assays were completed with assistance from Dr Thilipan Thaventhiran.

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To Ammu and Abbu who have always challenged me.

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To my beloved Nazmeen who has emboldened and tolerated me.

Abbreviations

11-HSD	11-Hydroxysteroid Dehydrogenase
17-OHP	17-Hydroxy Progesterone
ACTH	Adrenocorticotropic Hormone
Addi-QoL	Addison's Quality of Life Questionnaire
AI	Adrenal Insufficiency
APS	Autoimmune Polyglandular Syndrome
AUC	Area-Under-The-Curve
BD	Becton Dickinson
BSA	Body Surface Area
BMAL1	Brain–Muscle–Arnt-Like Protein 1
BMD	Bone Mineral Density
BMI	Body Mass Index
BNP	Brain Natriuretic Peptide
bpm	Beats Per Minute
BSA	Bovine Serum Albumin
CAH	Congenital Adrenal Hyperplasia
CBG	Cortisol Binding Globulin
CKD	Chronic Kidney Disease
CLOCK	Circadian Locomotor Output Cycle Kaput
cOC	Carboxylated Osteocalcin
CRF	Clinical Research Facility
CRH	Corticotropin Releasing Hormone
cRPMI	Complete RPMI
CRY	Cryptochrome Genes
CTX	C-Terminal Telopeptide
CV	Coefficient of Variation
DEXA	Dual Energy X-Ray Absorptiometry
DBP	Diastolic Blood Pressure
DKK1	Dickopf-1

DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EMA	European Medicines Agency
EU-AIR	European Adrenal Insufficiency Register
FBC	Full Blood Count
FBS	Foetal Bovine Serum
G-CSF	Granulocyte Colony Stimulating Factor
GH	Growth Hormone
GI	Gastrointestinal
GIO	Glucocorticoid Induced Osteoporosis
GNCQ	German National Cohort Questionnaire
GR	Glucocorticoid Receptor
HbA1c	Haemoglobin A1c
HDL	High Density Lipoprotein
HLA	Human Leukocyte Antigen
HOMA-%β	Homeostasis Model Assessment of β-cell Function
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
HPA	Hypothalamic-Pituitary Axis
HPLC	High Performance Liquid Chromatography
hs-CRP	High Sensitivity CRP
hs-Trop	High Sensitivity Troponin I
ICHNT	Imperial College Healthcare NHS Trust
IQR	Interquartile Range
ITT	Insulin Tolerance Test
ITU	Intensive Therapy Unit
IV	Intravenous
IVMP	IV Methyl-Prednisolone
LDL	Low Density Lipoprotein
LLOQ	Lower Limit of Quantification
MACE	Major Adverse Cardiovascular Outcomes

MAP	Mean Arterial Blood Pressure
MCSF	Macrophage Colony Stimulating Factor
MR-HC	Modified Release- Hydrocortisone
mRNA	Messenger Ribonucleic Acid
NIHR	National Institute for Health Research
NK Cells	Natural Killer Cells
NTX	N-Terminal Telopeptide
NT-proBNP	N-Terminal Pro- Brain Natriuretic Peptide
NWLP	North West London Pathology
OC	Osteocalcin
ODST	Overnight Dexamethasone Suppression Test
OGTT	Oral Glucose Tolerance Test
OPG	Osteoprotegerin
OMNI-AID Study	Objective Markers and New Indicators in Adrenal Insufficiency Disease Study
P1NP	Procollagen Type 1 N-terminal Propeptide
PAI	Primary Adrenal Insufficiency
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PER	Period Genes
PTH	Parathyroid Hormone
POMC	Proopiomelanocortin
RANK-L	Receptor Activator of Nuclear Factor κB Ligand
RPM	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute-1640 Medium
SF36	Short Form Health Survey 36
SAE	Serious Adverse Events
SAI	Secondary Adrenal Insufficiency
SBP	Systolic Blood Pressure
SCN	Suprachiasmatic Nucleus
SHS	Subjective Health Surveys

SMR	Standardised Mortality Ratio
SPC	Summary of Product Characteristics
SST	Short Synacthen Test
STAT5	Signal Transducer and Activator Of Transcription 5
T2DM	Type 2 Diabetes Mellitus
uOC	Undercarboxylated Osteocalcin
URTI	Upper Respiratory Tract Infection
UTI	Urinary Tract Infection
WBC	White Blood Cell Count
WHO	World Health Organisation
WHR	Waist-Hip Ratio

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Chapter 1: General Introduction

1.1 Adrenal Insufficiency

Adrenal Insufficiency (AI) is a condition characterised by the relative absence of glucocorticoid hormones. AI is treated by simply replacing the deficient hormone, but the condition is still associated with increased cardiometabolic risk and early mortality. In particular, individuals with the condition are twice more likely to die than the general population. The cause of this increase has not yet been fully explained, but it is clear that a possible solution may lie in improving hormone replacement regimens.

1.1.1 Pathophysiology

AI is the failure of the adrenal glands to produce sufficient quantities of cortisol, the principal stress hormone (1). Cortisol is a glucocorticoid made in the zona fasciculata of the adrenal gland. It is essential for life due to its actions that are not limited to: regulating plasma electrolyte levels, permitting renal free water excretion, co-ordinating an integrated stress response, and immunomodulation (2,3). Our fundamental understanding of the role of cortisol in the stress response is informed by Plumpton and Besser's seminal work investigating the effects of major surgery on cortisol secretion in healthy individuals (4). By performing insulin tolerance tests (ITTs) prior to surgery, the authors confirmed that individuals who demonstrate a cortisol rise of 150 nmol/L, with a peak greater than 550 nmol/L will predictably mount a larger and appropriate response to major surgery. As a result of this work, the cortisol values quoted continue to be used to define the cut-off values for ITTs and short synacthen tests (SSTs), which are the dynamic function tests necessary to confirm the diagnosis of AI.

AI is described as either primary adrenal insufficiency (PAI) or secondary adrenal insufficiency (SAI), depending on the level at which the hypothalamic-pituitary-adrenal (HPA) axis has failed (Figure 1.1). PAI, also known as Addison's disease, involves direct impairment of adrenal tissue to synthesise cortisol and aldosterone. The commonest global cause of this was previously tuberculosis infection of the gland, but in developed countries the leading cause is autoimmune adrenalitis (5). This may be isolated or in association with an endocrinopathy such as autoimmune polyglandular syndrome (APS) (1,6). Other rarer causes include: congenital adrenal hyperplasia (CAH), adrenal carcinoma, drug induced adrenalitis, adrenoleukodystrophy, adrenal haemorrhage, rarer infections such as histoplasmosis and adrenalectomies in treatment of other conditions. SAI is characterised by the inability of the anterior pituitary gland to produce adrenocorticotropic hormone (ACTH), a 39-peptide hormone which stimulates cortisol secretion (7). SAI is commonly caused by pituitary tumours, pituitary surgery, exogenous glucocorticoid suppression and less commonly, pituitary stalk inflammation and genetic conditions (1).

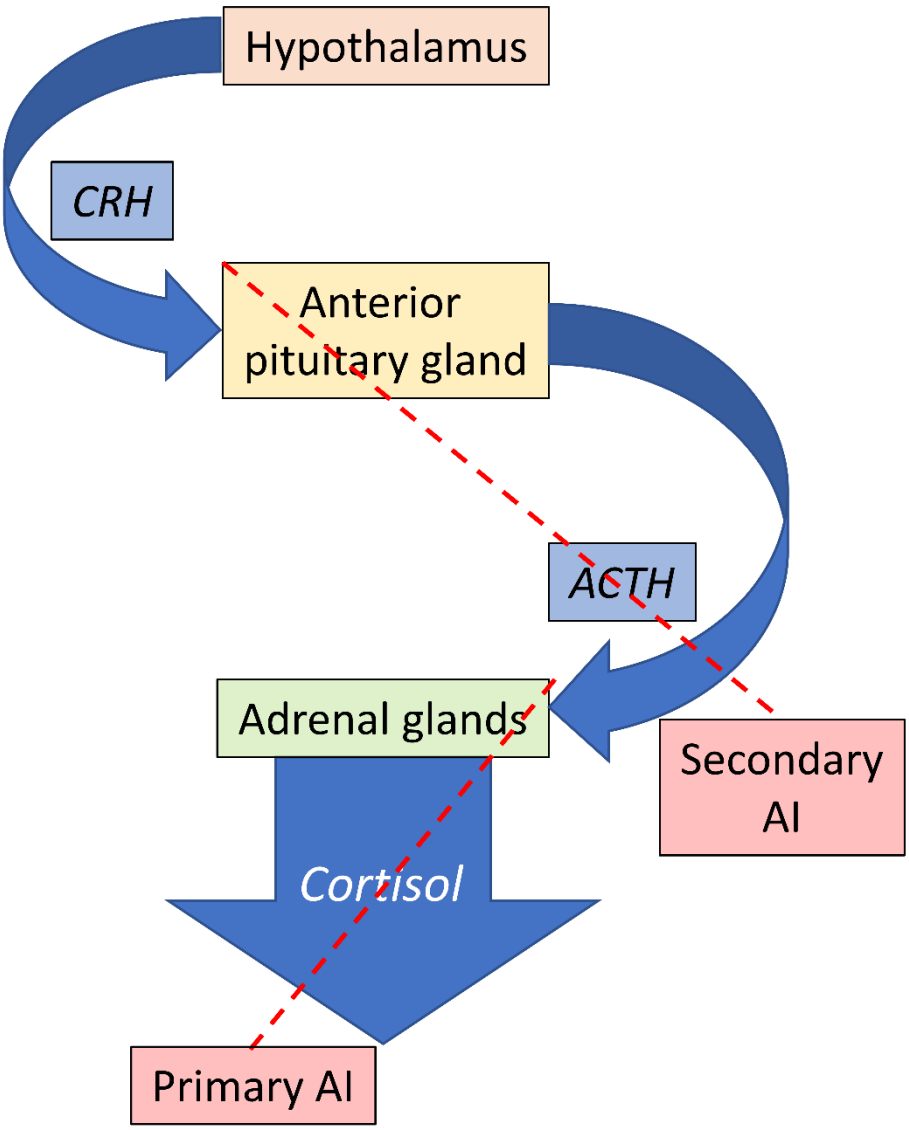

Figure 1.1- The hypothalamic-pituitary-adrenal (HPA) axis. Corticotropin releasing hormone (CRH) is secreted from the hypothalamus and stimulates adrenocorticotropin (ACTH) release from the anterior pituitary. ACTH stimulates cortisol secretion from the zona fasciculata of the adrenal glands. Failure of the adrenals to secrete cortisol is primary adrenal insufficiency (PAI). Failure of the anterior pituitary to secrete ACTH is primary adrenal insufficiency (SAI).

1.1.2 Symptoms and challenges of diagnosis

Most symptoms of AI are common between PAI and SAI. They include lassitude, anorexia, postural hypotension, hyponatraemia, nausea, vomiting, diarrhoea and weight-loss (5). These features specifically relate broadly to cortisol deficiency, although there is overlap with the physiological

actions of aldosterone. In PAI, patients may present with hyperpigmentation, particularly in the oral cavity and palms, hyperkalaemia or sequalae of related autoimmune conditions such as hypothyroidism, vitiligo or coeliac disease (1). The absence of cortisol-stimulated negative feedback provokes increased expression of the precursor, proopiomelanocortin (POMC), and in turn ACTH. Physiological cleavage of POMC and ACTH yields γ-melanocyte stimulating hormone (MSH) and α-MSH respectively, which in turn stimulate melanin production, causing hyperpigmentation although this is not always present (8,9). Destruction of adrenal tissue causes the inability to synthesise aldosterone, preventing renal excretion of potassium and renal reabsorption of sodium (9). The latter can cause hypovolaemic states that predispose to hypotension. SAI may be complicated by disturbances in other hormonal axes, causing reduced libido in men or amenorrhoea in females and diabetes insipidus (5). Mass effect from pituitary expansion can also cause headaches and bitemporal hemianopia (10). Acutely, patients may present with an adrenal crisis, a life-threatening hypotensive, hypoglycaemic, hyponatraemic, hypercalcaemic, hyperkalaemic state, which is commonly associated with nausea, vomiting and non-specific abdominal pain. Crises must be treated promptly to avoid mortality (11).

AI is notoriously difficult to diagnose because of its non-specific presentation (12). A German survey conducted in 2006 identified 216 responses from patients with PAI and SAI (13). Of the patients who did not have surgical cause of AI, 47% received a correct diagnosis within one year of symptoms and 20% of patients were diagnosed at greater than 5 years. Further, 67% of patients obtained opinions from at least three different physicians before a correct diagnosis was applied and 15% were misdiagnosed by their first physician. Taken together, this study demonstrates the significant burden that AI represents on both patients and the doctors who make the diagnoses.

1.2 Epidemiology

The prevalence of PAI has been estimated between 93-140 per million in European countries since the mid-1990s (14-17). There is an annual incidence of 5.6 to 6.2 per million per year (15,17). According to one of the largest epidemiological studies for PAI, which looked at 10 hospital registers serving 916,000 patients, the average age of men and women with PAI are 42 and 60 respectively (17). Males also tend to be younger at diagnosis, with a mean age of 29.2 versus 37.9 for females (18). There is limited data available outside of Europe. Data from Japan and South Korea indicates that the numbers may be far lower in Asia (19,20). In South Korea, the reported prevalence is 4.17 per million with an annual incidence of 0.45 per million per year (20).

SAI is more common, with the prevalence accepted to be 150-280 per million in European countries (1). There is limited data available as most studies broadly investigate pituitary pathology such as pituitary adenomas or hypopituitarism, as opposed to secondary hypoadrenalism specifically. Therefore, there are only two studies that are predominantly quoted (21). A UK-based study found 769 patients with corticotrophin deficiency requiring glucocorticoid replacement in the Birmingham area containing about 5.5 million people, corresponding to a prevalence of 140 per million (22). A further Spanish study reported 43 individuals in a population of 151,587, were diagnosed with corticotrophin deficiency, corresponding to a prevalence of 284 per million with an associated incidence that can be extrapolated to 26.2 per million per year (23). The average age of diagnosis of pituitary adenomas is 53.8 and 50.3 for males and females respectively, with a peak incidence during the sixth decade (24).

Glucocorticoid induced AI, is now more commonly referred to as tertiary AI. Patients with tertiary AI have the potential to reverse the condition with careful weaning of glucocorticoid regimens, and therefore have not been considered in depth. Due to poorly understood corticotroph atrophy, a number of tertiary AI patients require lifelong glucocorticoid replacement. An accurate estimate of the prevalence of this population has not yet been made. A systematic review of 73 studies has

highlighted that the median prevalence of AI in glucocorticoid treated individuals is 37.4% (25). After 3 years following glucocorticoid withdrawal, 15% still have AI. With data indicating that an average of 0.75% of the UK population has at some point received long-term glucocorticoids, defined as greater than 3 months, the number of patients with tertiary AI needing lifelong replacement is likely to be close to 75,000 individuals in the UK (26).

1.3 Mortality

In the absence of treatment, AI is quickly fatal. This was characterised by Dunlop in 1963 who described his experience of managing patients with Addison's disease over decades (27). Between 1928 and 1938, 29 of 34 patients with Addison's disease died within 2 years, and the remaining 5 died within 5 years. This corresponds to a 2-year and 5-year mortality of 85% and 100%. With the availability of glucocorticoid replacement therapy, initially with 11-deoxycorticosterone synthesised by Reichstein and colleagues in 1937 (28), and later cortisone acetate from 1948, the prognosis of Addison's disease had markedly improved with one individual surviving over 25 years. The advent of synthetic glucocorticoids prevented deaths from chronic adrenal failure and acute crises but has led to an era of liberal dosing which has inadvertently caused an increase in longer-term cardiometabolic death (29).

1.3.1 Current disparity in mortality

The disparity between patients with PAI and the general population has been best characterised in Norway, where data indicates a reduction in life expectancy (30). Men can expect a reduction of up to 11.2 years, whilst women show a reduction of up to 3.2 years compared to the general population. The same study did not demonstrate an increased standardised mortality ratio (SMR) when all Addison's patients are taken together. However, when patients under 40 were examined as a separate

cohort, their SMR was determined to be 1.5 and was significantly higher than healthy individuals. Cardiovascular disease, adrenal failure and cancer were the top three causes of death.

A retrospective observational study in Sweden collected data from the National Hospital and Cause of Death registers between 1987 and 2001 (31). The ability to interrogate well maintained national databases allows for Swedish studies to provide particularly good insights into patient outcomes. Data from patients with Addison's disease was compared to the general population. The relative risk of mortality for men with Addison's disease was 2.19, and 2.86 for women. The study discovered that cardiovascular disease, and specifically ischaemic heart disease was the leading cause of death. This was followed by malignancy, endocrine causes, respiratory causes and infectious diseases. Compared with the general population, patients with Addison's show an increased SMR of 2.7 in a subsequent Swedish study (32). The SMR was more favourable for males with Addison's at 2.5, compared to 2.9 for females. Cardiovascular disease continued to be the most common cause of death in 43% of individuals, followed by malignancy in 20.5%.

The increased long-term mortality in hypopituitary and SAI patients has been recognised for decades (33). As patient with SAI are likely to have deficiencies in other hormonal axes, it has proven difficult to show the excess mortality is specifically because of cortisol deficiency or subsequent treatment as opposed to being multifactorial. In a cohort of patients with growth hormone (GH) deficiency, there is a 3.8x increase in the number of deaths compared to those expected at any timepoint (34). The presence of concurrent AI however, confers a 7-fold increase in the risk of death.

Reviews on the subject of mortality in hypopituitarism have concluded that there is indeed an increased burden of cardiovascular disease and malignancy, although data on the latter is conflicting (35). Conclusions about increased malignancy in SAI patients tends to be data that has been extrapolated from the aforementioned PAI focussed studies. More recently, the European Adrenal

Insufficiency Register (EU-AIR), which was set up to monitor the long-term safety of modified release hydrocortisone (MR-HC), has been collecting data from hospitals in Germany, the UK, Netherlands and Sweden (36). The registry includes data from patients with SAI, as well as PAI. In this group, patients with SAI were noted to have a greater mortality of 1.5%, compared to 1.0% for patients with PAI (37). The most common cause of death was cardiovascular disease, accounting for 35% of deaths.

1.3.2 Adrenal Crises

Adrenal crises in AI are a cause of premature death. In Norwegian populations, adrenal failure accounts for approximately 15% of deaths and was second only to cardiovascular disease (30). Retrospective, patient reported data from the UK indicates that 8% of individuals with AI have needed hospital treatment for a suspected crisis in the previous year and vomiting or diarrhoea are the commonest triggers (38).

A prospective German study followed up PAI and SAI patients for two years with 6-monthly subjective health surveys (SHS) and questionnaires, after being educated on emergency glucocorticoid dosing or "sick day rules" (39). From 423 participants, baseline data indicated that there were 15 episodes of emergency treatment per 100 patient-years, dropping to 8.3 crises per 100 patient-years after education, with an episode mortality rate of 6.3%. A further retrospective Swiss study reported 4.4 adrenal crises per 100 patient-years (40). The most common precipitant was gastroenteritis in both studies.

Japanese data from 2007 to 2014 showed that adrenal crises account for a very small proportion of global inpatient admissions, with 799 cases admitted from a total of 34 million admissions (41). Inpatient mortality during a crisis was 2.4%, which is lower than demonstrated in European studies. Of note, infections were the leading precipitant, complicating 15% of crisis admissions. Gastroenteritis

was the third leading cause, complicating 8.5% of admissions. It disproportionately affected individuals with PAI versus SAI, causing 12.9% and 6.5% of admissions respectively. Speculative explanations for this difference may involve the inability of PAI patients to produce mineralocorticoids, which may confer a protective effect. Conversely, patients with SAI were significantly more like to be admitted to intensive therapy units (ITUs). The reason for this was not explored, but the older age of SAI patients may be responsible.

It is apparent that there is discordance in the reported figures of the adrenal crisis frequency. In recent times, it may be as uncommon as 4.1 events per 100 patient-years in treated patients (42). Overall estimated mortality because of a crisis stands at 0.5 per 100 patient-years (11). There seems to be a trend towards lower event rates in the more recent past, which could represent better recognition and treatment of the condition. In studies which differentiate between crisis events in patients with PAI and SAI, the event rate is always higher in PAI (11). Whilst the physiological basis of this has not been elucidated, mineralocorticoid deficiency is an obvious research target. Although the prevention of adrenal crises is an important concern, the consensus derived from the available data is that crises are unlikely to be the most significant cause of excess mortality in the AI population.

1.4 Treatment

The mainstay of management is hormone replacement. In the case of PAI, glucocorticoid and mineralocorticoid replacement therapy are required (43). The latter is achieved using fludrocortisone tablets once daily. In SAI, only glucocorticoid replacement is indicated.

The evolution of treatment is closely tied to the ability to synthesise different adrenal glucocorticoids. The first widely available pharmaceutical treatment was 11-deoxycorticosterone in 1937 (44). The inadvertent isolation of cortisone from bovine adrenal extracts in the 1930s and 1940s would be

developed into a successful treatment for Rheumatoid Arthritis in 1948 (45,46). The experimental use of cortisone would lead to the award of the Nobel Prize in physiology or medicine and opened the door to the synthesis of other synthetic glucocorticoids such as hydrocortisone and prednisolone.

1.4.1 Guidelines for management

There are several active guidelines for the diagnosis and management of AI, which have broadly remained consistent since 2010 (47). There are no UK-specific guidelines at present and development of the NICE guidelines has been suspended as of November 2016, awaiting the completion of relevant studies (48). The Endocrine Society Clinical Practice Guidelines, published in 2016, are used widely in the UK and Europe to inform practice as a surrogate (49). The authors advocate hydrocortisone 15 mg to 25 mg or cortisone acetate 20 mg to 35 mg in two to three divided doses, as first line glucocorticoid replacement therapy. Prednisolone is recommended as an alternative at doses of 3 mg- 5 mg daily, as a single dose, or in two divided doses. Mineralocorticoid replacement should be accomplished with fludrocortisone initially with 50 µg to 100 µg.

In other regions, guidance on preferred medication does vary. In China, it is reported that the first line treatment is prednisone (50). In Japan, hydrocortisone is favoured in twice or thrice daily regimens, with prednisolone only mentioned in the context of pregnancy and CAH (43). It is unclear whether the variation between regions is fully evidence based, or because of the relative availability of different drugs in different regions. For instance, the dependence on cortisone acetate in Italy has historically been because of a lack of hydrocortisone availability (14).

Hydrocortisone and prednisolone have been explored below, as they are central to the present study. Cortisone has been reviewed briefly for interest, as it is being slowly phased out of use. The current

body of research on MR-HC has warranted inclusion as it has contributed significantly to the understanding of circadian regulation and immune response to glucocorticoids.

1.4.2 Hydrocortisone and cortisone

Both hydrocortisone and cortisone were initially produced by extraction from animal adrenal tissue in the 1930s to 1940s and their histories are entwined (51). A pharmaceutical process to produce both hormones was only developed in the late 1940s. The initial focus was on cortisone acetate, a more potent ester of cortisone, following seminal case reports of its ability to reverse the symptoms of rheumatoid arthritis (45,52). Until 1951, cortisone was believed to be the principal adrenocortical hormone when it was discovered that greater quantities of hydrocortisone, later recognised to be cortisol, was produced by human adrenal glands than cortisone in response to ACTH administration (53). Soon after, it was discovered that hydrocortisone is more potent than cortisone (54).

Cortisone is a precursor to hydrocortisone, and on administration is converted to hydrocortisone by 11-hydroxysteroid dehydrogenase (11-HSD) type 1 in the liver as part of first pass metabolism (55). The conversion of cortisone to hydrocortisone is variable, with the bioavailability estimated to be between 50% and 80% (56,57). Based on a study comparing intravenous (IV) 50 mg hydrocortisone to oral 50 mg hydrocortisone and oral 50 mg cortisone acetate in 10 individuals, where the bioavailability of cortisone acetate was 80% of the values seen with oral hydrocortisone, it is now widely accepted that the conversion ratio for hydrocortisone to cortisone is 1:0.8 (58).

Hydrocortisone has a half-life of approximately 2 hours (59,60). Whilst there is no data on the terminal half-life of orally administered cortisone, it is fair to assume that the current divided dose regimens are unlikely to saturate hepatic 11-HSD activity and that the terminal half-life will be similar to hydrocortisone. As a result of the short half-life, both hydrocortisone and cortisone must be

administered in multiple daily doses. Although it was initially thought that twice daily regimens were sufficient (61), by 1988 it had become apparent that twice daily regimens would lead to a cortisol nadir in the afternoon with a trend towards reduced wellbeing scores at the same time (62). Thrice daily regimens have since been adopted (63).

Worldwide, hydrocortisone and cortisone acetate are used by 75% and 6% of AI patients respectively (64). A study of a primary care database revealed that in the UK, 72% of patients receive hydrocortisone, but data on cortisone was not reported (65). Data from the EU-AIR registry, which includes the UK in the context of other European countries, found that hydrocortisone use was even more widespread at 87% with cortisone acetate prescribed for only 4% (66). The majority of patients receiving hydrocortisone are prescribed 20 mg to 25 mg daily in divided doses. This accounts for 42% of AI patients taking hydrocortisone. The most common regimen is twice daily, used by 48% of patients, compared to 43.6% who take their tablets thrice daily. It is unclear whether the preference for twice daily regimens is due to reduced adherence with more frequent regimes.

Despite advocacy for tailoring doses to individuals, in practice most patients are started on a one-size-fits-all hydrocortisone regimen of 10 mg in the morning, 5 mg at noon and 2.5 mg or 5 mg in the afternoon based on an early analysis of hydrocortisone day curves (63). The less common availability of hydrocortisone tablets at preparations less than 10 mg is a further obstacle to personalisation (67). There is body of evidence indicating that most regimens of hydrocortisone lead to inadequate dosing (68). Compared to 60 healthy controls, 50 French patients with AI demonstrated cortisol values at 8am, 4pm and midnight that were outside the expected range. Whilst receiving a mean dose of 25 mg of hydrocortisone in divided doses, 11%, 13% and 31% of patients respectively, had suboptimal levels of cortisol at the defined timepoints. Additionally, 68%, 42% and 14% demonstrated levels that were excessive at the same timepoints.

Dose customisation has been suggested. Based on an assessment of 20 AI patients who received hydrocortisone at fixed doses of 10 mg, body surface area (BSA) adjusted doses at 5.5 mg/m^2 and weight-based doses, recommendations for personalising the morning dose of hydrocortisone to 0.12 mg/kg were developed (69). More recently, pharmacokinetic profiles of thrice daily hydrocortisone administered at 14 mg/m^2 and 10 mg/m^2 demonstrated excess corticosteroid exposure as measured by cortisol area-under-the-curve (AUC), when compared to controls (70). When dosage was reduced to 6mg/m^2, cortisol profiles proved to be more comparable to healthy controls, in 81.5% of patients. Whilst the results of this study are consistent with growing evidence that most hydrocortisone regimens result in over-replacement, they remain in excess of measured daily adrenal cortisol production rates, which has been estimated consistently at 11.9 mg/day to 18.7 mg/day (71,72).

The original shift from cortisone acetate to hydrocortisone was sparked by observations of the variable bioavailability of cortisol after cortisone administration and concerns that it was not a reliable means to achieving reproducible levels of cortisol in the blood (73,74). There have however been observations that patients prescribed cortisone acetate do show improved glycometabolic profiles with a significantly lower glycated haemoglobin (HbA1c) versus patients receiving hydrocortisone (75). A study which examined a Swedish population who had switched from cortisone acetate to hydrocortisone using a ratio of 1:0.8, comparing them to patients who remained on cortisone, found that patients who switched experienced significant weight gain, with concurrent increases in waist circumference and fat mass, measured by dual energy X-ray absorptiometry (DEXA) scanning (76). Worsening of diastolic blood pressure (DBP) and HbA1c was also associated with switching to hydrocortisone. Whilst it is possible that the results of this study may have been influenced by patients who remained on cortisone acetate, starting on a lower dose of 21.6 mg per day versus 26.6 mg in the switch group, it is also likely that patients who remained on cortisone were protected against a tendency for over-replacement with hydrocortisone by its reduced and variable bioavailability thereby ameliorating excess glucocorticoid exposure.

1.4.3 Prednisolone and prednisone

Prednisolone and prednisone were first synthesised by Arthur Nobile for the Schering Corporation in 1950, as an anti-inflammatory arthritis treatment (77). The basic chemical structure of cortisol and cortisone are conserved in prednisolone and prednisone respectively, with the exception of a double bond between carbon-1 and carbon-2, in the first tetracyclic ring (Figure 1.2). It is owing to this modification that prednisolone has an extended half-life of 3.2 hours and prednisone has a half-life of 3.3 hours (59). Prednisolone has a relative molecular mass of 360, compared to prednisolone which has relative molecular mass of 358. Prednisolone is the biologically active form and is synthesised with the first pass metabolism of prednisone, the handling of which uses the same pathways as cortisone. Specifically, prednisone is converted to prednisolone by hepatic 11-HSD1 with a bioavailability of 78% to 86% (78). Given the high bioavailability of prednisolone following prednisone administration, both glucocorticoids will be considered equivalent to each other for the purposes of literature review in this thesis. Preference for prednisolone over prednisone in different regions is predominantly determined by the marketing of each drug. For example, prednisone is favoured in the USA and prednisolone is used in the UK (67,79). The participants in the present study were managed specifically with prednisolone.

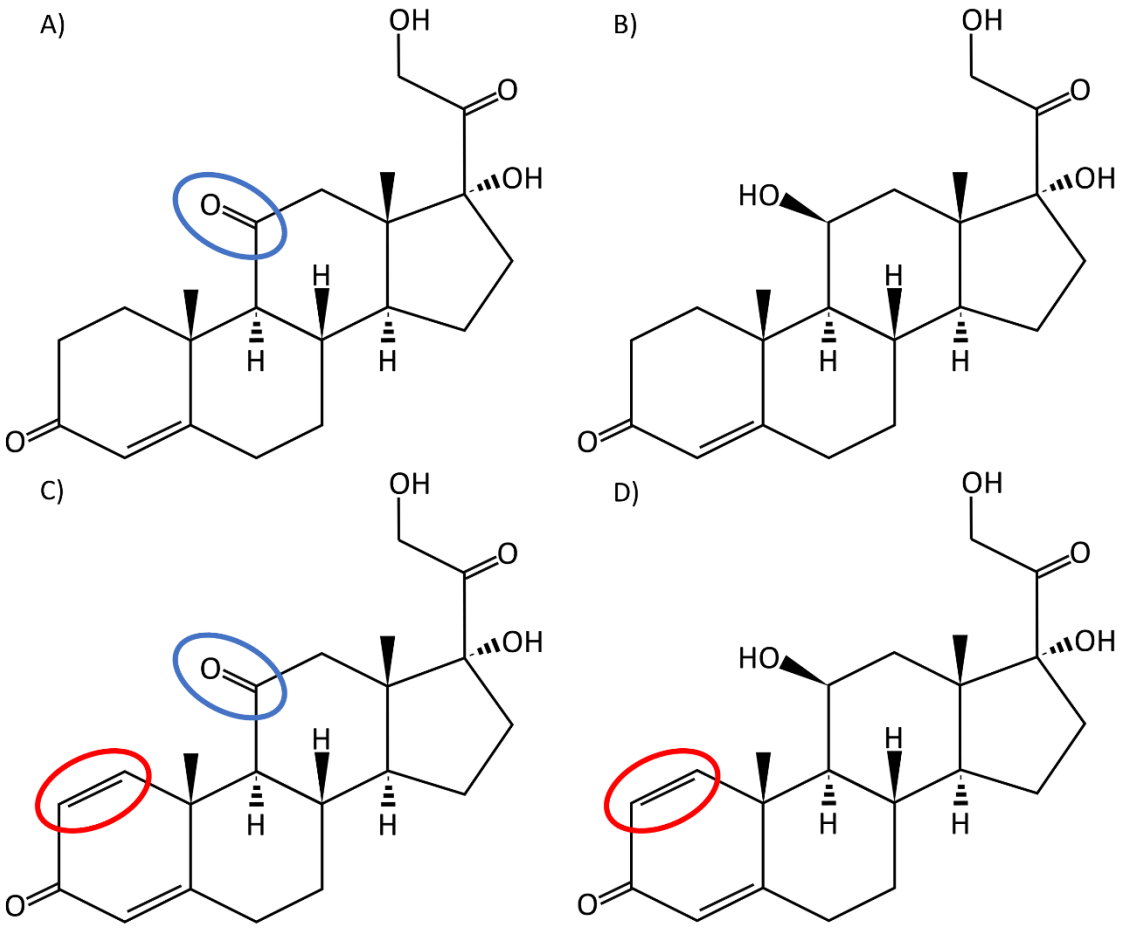

Figure 1.2- Variations of chemical structure of selected glucocorticoids. A) Cortisone B) Hydrocortisone/cortisol C)Prednisone D)Prednisolone. Both prednisone and prednisolone are equivalent to cortisone and hydrocortisone respectively. The double bond between carbon-1 and carbon-2 (circled-red) confers both molecules with a longer half-life. The conversion of a ketone group to a hydroxyl group on carbon-11 (circled-blue) differentiates hydrocortisone from cortisone and prednisolone from prednisone, making the latter molecules of both pairs, biologically inert.

Prednisolone is more potent than hydrocortisone, showing 2.26-fold greater affinity for the glucocorticoid receptor (GR) (80). Additionally, it binds more avidly than cortisol, with cortisol binding globulin (CBG) and albumin, although can be displaced by cortisol in a non-competitive manner, when both glucocorticoids are present (81). There is also evidence that prednisolone binds with the GR for longer periods than cortisol before dissociation, leading to differences and delays in cessation of downstream nuclear transcription (82). The combined effects of these characteristics are unclear,

however the bioequivalence of hydrocortisone to prednisolone has been consistently assumed to be 1:4 for over 65 years (67,83). This conversion ratio was derived by comparing the anti-inflammatory effects of prednisolone and hydrocortisone but cannot be critically reviewed as the original data was presented at a conference and never published (83).

There are no end organ markers that have been discovered in AI that would allow physicians to gauge the adequacy of glucocorticoid replacement. Studies in populations with CAH do however provide valuable insights as markers such as growth velocity and androgen levels provide an indication about the correct levels of dosing. A study involving 9 children, 6 of whom had CAH and 3 with hypopituitarism or AI, investigated the effect of switching between different glucocorticoid regimens (84). When the patients were switched according to a ratio of 1:5, from a mean hydrocortisone dose of 17.6 mg/m^2 in three divided doses to 3.6 mg/m^2 of prednisolone in two divided doses, the children experienced a significant reduction in height standard deviations and growth velocity after 6 months. The patients with CAH saw a suppression of 17-hydroxy progesterone (17-OHP) from 41.6 nmol/L to 0.9 nmol/L. Prednisolone doses were reduced over 3-month periods, and the measured parameters normalised when doses were adjusted to a bioequivalence ratio of approximately 1:15.

A more robust Brazilian trial involved 44 individuals with CAH who were assigned to receive either hydrocortisone three times daily at a total dose of 10-15 mg/m^2 or prednisolone once daily at 2.4-3.75 mg/m^2 (85). Equivalence of hydrocortisone to prednisolone was initially taken to be 1:4. During the year-long study, the authors reported that the dose of prednisolone used was excessive based on clinical and biochemical monitoring. Patients receiving prednisolone required tapering down to a final dosing of 1.8-3.0 mg/m^2, compared to 12-20 mg/m^2 of hydrocortisone used at the end. The researchers concluded that based on their experience, the bioequivalence ratio of hydrocortisone to prednisolone is currently understated, and that the true ratio is in fact 1:6-8.

Whilst it may hold true that at anti-inflammatory doses the conversion rate between hydrocortisone and prednisolone is 1:4, there is mounting evidence that this may not be correct when considering the lower doses used for AI and CAH. The existing evidence base for the use of prednisolone in AI is conflicted with discordant outcomes. This is further complicated by the use of the 1:4 ratio when calculating hydrocortisone equivalent doses. For example, one study compared 21 PAI patients receiving 30 mg total daily hydrocortisone to 9 PAI patients taking prednisone 7.5 mg once daily (86). Each patient had biochemical bone markers including osteocalcin (OC) quantified, and a DEXA scan to measure bone mineral density (BMD) at baseline and one year. No significant differences between the groups were detected, although the authors did comment that the BMDs in the prednisone group were lower and the prevalence of osteoporosis was higher but without achieving significance.

A more recent study compared patients receiving 7.5 mg of prednisone once daily to patients receiving hydrocortisone, receiving a mean of 28 mg daily in divided doses (87). The authors noted that the prednisone group contained a significantly greater proportion of patients with osteoporosis than the hydrocortisone group, and that the prednisone group had lower BMD scores although this was not statistically significantly different. These studies seek to compare 7.5 mg of prednisone with approximately 30 mg of hydrocortisone, assuming equivalence. Such analysis does not take into account that prednisone is in fact more potent and that 7.5 mg of prednisone may be more equivalent to 45 mg to 60 mg of hydrocortisone. The effects on BMD and presence of iatrogenic osteoporosis can simply be explained by the patients in the studies receiving excess glucocorticoid, as opposed to a prednisone specific effect. This pattern of increased bone loss with greater glucocorticoid exposure has already been demonstrated with hydrocortisone, where increasing doses of hydrocortisone between 15 mg and 30 mg cause dose-dependent suppression of bone turnover as detected by biochemical markers (88).

The body of evidence for the use of prednisolone in AI, is broadly negative but complicated by the use of excessive doses in the order of 7.5 mg once daily in most cases, and rarely doses at the lowest of 5 mg once daily. It has become apparent at Imperial College Healthcare NHS Trust (ICHNT) that the doses of 5–7.5 mg of prednisolone used in the past, were too high. Since 2014, the prevailing practice has been to use low-dose prednisolone in the order of 2 mg-4 mg in most cases (89). This has been corroborated by other groups, who are using doses of prednisone that are as low as 1 mg-2 mg daily in adrenalectomised patients with PAI (79). As this practice is relatively new, there is a paucity of evidence comparing low-dose prednisolone with hydrocortisone in AI.

With the introduction of a mass spectrometry assay at ICHNT, it is possible to offer greater individualisation of prednisolone doses by measuring 8-hour trough prednisolone levels and tailoring doses so that patients are able to achieve a target concentration of 15-25 µg/L (90). Although quantification of prednisolone levels was possible as early as in the 1970's, the high performance liquid chromatography (HPLC) methods did not offer sufficient sensitivity to be able to quantify levels below 25 µg/L (91). The practice of performing trough levels further encourages lower glucocorticoid exposure in patient groups and is a break from the routine use of one-size-fits-all doses of hydrocortisone.

Prednisolone use for glucocorticoid replacement is growing in popularity. In 2012, it was reported that 11% of AI patients were prescribed a prednisolone or prednisone regimen, of whom, 30% were receiving a once daily regimen and 53% on a twice daily regimen (64). By 2019, this has grown to 26% of patients receiving prednisolone in the UK (65).

1.4.4 Modified release hydrocortisone (MR-HC)

There are currently two preparations of MR-HC, Chronocort and Plenadren.

Chronocort is an enteric coated tablet which contains a sustained release layer, and a further hydrocortisone coated microcrystalline layer underneath, all of which is released in the small intestine (92). The tablet is administered at night to recreate the physiological rise in cortisol levels seen in the early morning (93). Early evidence suggested that administration of the drug at night, followed by a further tablet in the morning, could mimic the diurnal cortisol rhythm better than immediate release hydrocortisone, showing promise for the management of AI and CAH (94). Although further development has not continued for its use in AI, Chronocort was successful in Phase 2 studies, showing normalisation of serum androstenedione AUC, and suppression of urine androgen metabolites in patients with CAH (95,96). Despite concerns about Chronocort in 2018 after it did not meet it primary outcomes in Phase 3 studies, there is now a marketing authorisation application with the European Medicines Agency (EMA) indicating that it may soon be available for management of CAH (97,98). As the clinical trials involving Chronocort are designed for drug development and do not further the understanding of corticosteroid metabolism, it will not be discussed further.

Plenadren contains immediate-release and sustained-release hydrocortisone in a dual-release formulation (99). It is designed to be a once-daily preparation of hydrocortisone which provides a smoother and more physiological cortisol profile compared to immediate release hydrocortisone (100). Currently, only 5 mg and 20 mg tablets can be prescribed and these cannot be split, limiting doses to denominations of 5 mg (67). Between 2010 and 2016, Plenadren accounted for only 1.7% of glucocorticoid prescriptions for AI management in the UK (65). Plenadren has shown superiority to immediate release hydrocortisone with significant improvements in blood pressure and HbA1c noted in patients who undertook an open-label randomised crossover study (101). Sixty-four individuals with PAI took both Plenadren and thrice-daily hydrocortisone for 3 months at a time, Plenadren was associated with a significant reduction in weight by 0.9 kg, in systolic blood pressure (SBP) and DBP by 5.5 mmHg and 2.3 mmHg respectively, and HbA1c by 0.1%. Taken together, there is improvement in

cardiometabolic profile with Plenadren. The modified-release preparation may however expose patients to the risk of hospitalisation when afflicted by gastroenteritis as seen in 11 of 19 patients who experienced serious adverse events (SAE) in an 18-month extension to the above study (102,103).

In a cohort of 19 PAI patients receiving stable glucocorticoid replacement with 20 mg immediate-release hydrocortisone for at least 6 months, improvements in body weight, body mass index (BMI) and waist circumference was seen with 12 months of Plenadren treatment (104). There were significant improvements in low density lipoproteins (LDL) and in total cholesterol, although high density lipoproteins (HDL) and triglycerides were unchanged. Further studies have shown that these benefits are not limited to patients with PAI. A group of 49 patients included 36 with SAI, who were switched from baseline cortisone acetate or immediate-release hydrocortisone to 36 months of Plenadren, showed significant improvements in BMI, waist circumference and HbA1c (105). Patients diagnosed with pre-diabetes, were in particular, noted to have improved insulin sensitivity as measured by the Matsuda Index, in conjunction with lower insulin levels and AUC-insulin to 2 hours. These results are in keeping with more recent findings that Plenadren can help to reduce hepatic steatosis based on ultrasound parameters, after 12 months of treatment in SAI cohorts (106).

In addition to the clear trend of cardiometabolic benefits associated with Plenadren compared to thrice-daily immediate-release hydrocortisone, there is data to suggest that the advantages may also extend to bone health. In the earliest studies, it has been reported that Plenadren causes a significant increase in procollagen type 1 N-terminal propeptide (P1NP), a bone formation marker, after just 3 months of treatment compared to immediate-release hydrocortisone (101). DEXA imaging on 14 SAI patients in a retrospective study has confirmed that improvements in BMD after 2 years of Plenadren treatment (107). Patients had been diagnosed with AI for median of 10 months, having received stable immediate-release hydrocortisone or cortisone acetate replacement for at least 1 year with baseline

DEXA scan. They showed 10%, 11.5% and 3.1% increases in lumbar spine, femoral neck and total hip BMD, respectively from baseline.

The DREAM study has proven to be of key importance. A mixture of 89 PAI and SAI patients taking stable baseline multidose regimens of hydrocortisone or cortisone for at least 3 months, were split into two groups (108). One group of 43, were randomised to continue on their usual glucocorticoid replacement regimen. Most of these individuals were receiving cortisone acetate at relatively high equivalent doses. The second group of 46 switched onto open-label Plenadren once-daily. The study included an additional 25 healthy volunteers as a control comparator group. Patients continued on their allocated treatment for 24 weeks, with data collected at baseline, 12 weeks and 24 weeks. Although the primary outcome was bodyweight change, the group collected peripheral blood mononuclear cells (PBMCs) at the three timepoints and analysed the change in different immune cell populations. There was a significant weight reduction of 4.0 kg in the switch group with concurrent 1.7 kg/m^2 reduction in BMI, and 2.5 cm waist circumference loss. Glycometabolic risk factors also demonstrated encouraging trends, with a 0.3% reduction in HbA1c, although fasting glucose and insulin secretion indices were unchanged.

At baseline, the AI patients were noted to have higher numbers of classical (CD14+/CD16-) monocytes and lower numbers of nonclassical (CD14-/CD16+) monocytes and mature CD16+ natural killer (NK) cells compared to healthy volunteers. The former monocytes are predominantly proinflammatory phagocytic cells involved in the innate immune response, whilst the latter two populations are understood to be involved in maintaining vascular endothelia, defence against viral infection and surveillance. Whilst the patients who continued their baseline therapy and the healthy volunteers did not see any change in the size of these immune populations, the patients who commenced on Plenadren did. At 12 weeks and 24 weeks, the Plenadren patients showed significant decreases in their classical monocytes and increases in their nonclassical monocytes and mature NK cells, towards

the levels seen in the healthy volunteers. Concurrently, the group receiving Plenadren showed a reduction in the number of reported viral illnesses, which showed a significant association with the changes in the immune cells.

The DREAM study is the first to combine clinical outcomes with detailed immune cell population analysis (109,110). In doing so, it has furthered our understanding of the mechanistics behind increased infection rates in AI patients and may have inadvertently offered a way to assess the adequacy of replacement regimens with an objective measurable marker (111,112).

1.5 Causes of the Disparity in Mortality

With glucocorticoid replacement therapy, the mortality of patients with AI is improving and approaching that of the general population. The days of adrenal crises causing significant death appear to be in the past with numbers of crises reducing and other causes such as cardiovascular disease and malignancy becoming the most common causes of death (31). There is still a significant mortality gap between the disease population and their counterparts in the general population, the cause of which is unknown. The theories for the basis of this disparity in mortality, centre on the different unphysiological aspects of oral glucocorticoid replacement.

1.5.1 Insights from Autonomous Cortisol Secretion

Autonomous cortisol secretion is distinct from Cushing's syndrome as the glucocorticoid excess is mild to moderate and not overt, as in the latter (113). Of 365 patients with an adrenal incidentaloma in a Swedish study, 128 individuals were determined to have suspicion of autonomous cortisol secretion, and 33 a confirmed diagnosis of autonomous cortisol secretion, determined from the results of a 1 mg overnight dexamethasone suppression test (ODST) where 9am cortisol of 51 nmol/L to 138 nmol/L was suspicious and >138 nmol/L was a confirmed diagnosis (114). Two-hundred and four individuals

were diagnosed with normal cortisol secretion, demonstrating cortisol suppression of <50 nmol/L after an ODST. Whilst there were 7.8% deaths after a mean of 3.9 years in the normal cortisol secretion group, there were 11.7% deaths after 3.2 years and 18.2% after 2.9 years in the suspicious and diagnosed autonomous cortisol secretion groups respectively. Further analysis revealed that there was a significant relationship between the excess mortality and the size of the cortisol response post ODST.

There have been similar findings in an Italian study where, of 198 patients with incidentalomas, 129 were non-secreting, 59 had suspected autonomous cortisol secretion and 10 had confirmed autonomous cortisol secretion according to the abovementioned ODST criteria (115). After a mean follow-up of 7.5 years, there were significantly more patients with cardiovascular disease in the autonomous cortisol cohorts versus the normal secreting group at 16.7% and 6.7% respectively. Mortality was significantly higher in the secreting groups at 43.0% compared to 8.8% in the normal cortisol secreting cohort, again showing a significant interaction with post-ODST cortisol levels. Cardiovascular disease caused 48% of deaths compared to 43% for malignancy.

In a further UK-based retrospective study, it was apparent that the reduction in life expectancy in the study due to autonomous cortisol secretion was 4 years and 10 years in males and females respectively (116). Cardiovascular disease and infective conditions accounted for 50% and 33% of deaths respectively, which is higher than the 31% and 14% expected from national figures. It is clear that there are similarities between autonomous cortisol secretion and AI in the reduction of life expectancy seen (3.2 and 11.2 years for females and males respectively in AI) and the proportion of individuals afflicted or killed by cardiovascular disease (30). Taken together, the evidence indicates that the common pathophysiological characteristics of autonomous secretion and glucocorticoid replacement in AI must be considered. Common pathogenic mechanisms are the diminutive excess of cortisol exposure to individuals and the inherent loss of diurnal cortisol rhythmicity (117).

1.5.2 Excess Glucocorticoid Exposure

Data from the EU-AIR registry has shown that there is greater mortality in SAI cohorts in Europe compared to PAI cohorts at 1.5% and 1.0% respectively (37). In addition to the deceased patients being significantly older than those who survived, it was noted that they were more likely to have been diagnosed with type 2 diabetes mellitus (T2DM) and hypertension. This is also in keeping with the deceased patients receiving greater doses of hydrocortisone, at 24.0 mg daily compared to the surviving patients who received 19.3 mg daily.

UK data on 501 patients with acromegaly also demonstrated that whilst all acromegalic patients have an elevated SMR of 1.7, patients receiving hydrocortisone were at particular risk (118). There is a significant positive correlation between hydrocortisone daily dose and SMR, with patients receiving doses of 30 mg or greater showing a 2.9-fold increase in the relative risk of death compared to euadrenal patients with acromegaly in the cohort. Patients receiving 25 mg to 30 mg of hydrocortisone could expect a 1.6-fold increase in mortality risk, whilst there was no evidence of a significant change in risk at doses less than 25 mg.

More recently, a retrospective analysis of patients with non-functioning pituitary adenomas and SAI, stratified the SMR of patients according to the amount of daily hydrocortisone equivalent glucocorticoid medication they were receiving (119). Patients receiving greater than 20 mg showed an increased mortality hazard ratio of 1.88 compared to patients not requiring glucocorticoids. This was not the case for patients receiving less than 20 mg.

In a French cohort of PAI and SAI patients, those with dyslipidaemia were noted to be receiving significantly higher doses of hydrocortisone than those without dyslipidaemia (120). In PAI patients,

individuals with dyslipidaemia were receiving 28.1 mg of hydrocortisone compared to 27.2 mg in those without dyslipidaemia. The difference was more pronounced in the SAI cohort, where those with dyslipidaemia were in receipt of 20.8 mg versus 19.0 mg for those without. Similar findings were seen in the hypertensive subgroups of SAI patients, in all indicating that patients on higher doses of hydrocortisone were at greater risk of cardiovascular comorbidities.

This evidence suggests that the most common regimens of glucocorticoid replacement are associated with increased longer-term mortality and that mortality increases with escalating dosing regimens (121). The context of these studies, considering the data available from research into autonomous cortisol secretion, intimates that these observations may be because of AI patients receiving too much glucocorticoid replacement, even though the excess is likely to be very small.

In further support of this theory, there is data supporting the benefits of reducing hydrocortisone dosing regimens in patients. One open label crossover study invited participants with SAI to trial 6 weeks of three different daily hydrocortisone regimens of 30 mg, 20 mg, and 15 mg in two split doses (122). Ambulatory 24-hour blood pressure was measured on all participants and an ambulatory arterial stiffness index score was calculated. The study found that the 15 mg hydrocortisone regimen caused significantly lower arterial index stiffness scores compared to the other regimens, indicating a reduced cardiovascular risk.

The acute effects of increasing glucocorticoid dose and exposure has also been investigated. In a randomised, double blind crossover study, 47 SAI patients received 10 weeks of 0.2- 0.3 mg/kg of hydrocortisone in split doses and a doubled regimen of 0.4- 0.6 mg/kg in another 10-week period (123). This is in the context of previous weight based guidance advocating 0.22 mg/kg daily, in the past (69). Use of the higher hydrocortisone dosing was noted to cause a significant increase in SBP and DBP

of 5 mmHg and 2 mmHg respectively. There were also biochemical sequalae including lowering of plasma potassium, aldosterone and renin concentrations.

Another prospective study involved 17 patients with SAI, taking 30 mg of hydrocortisone equivalent glucocorticoid replacement for 7 days (124). The participants had been stably replaced for at least 6 months using different regimens including prednisolone, hydrocortisone and cortisone with a mean hydrocortisone equivalent daily replacement dose of 17 mg. Surrogate cardiovascular risk indicators from imaging and glycaemic response to a standard oral glucose tolerance test (OGTT) were recorded. Although no significant results were obtained for glycaemic handling and insulin secretion parameters, there was evidence of increased cardiovascular risk on the higher dosing regimen as evidenced by pulse wave analysis and assessment of endothelial function.

The literature available on Plenadren has broadly shown that converting patients from immediate-release hydrocortisone to Plenadren leads to a multitude of cardiovascular and glycaemic benefits. Whilst the improvements may be due to a more physiological replacement regimen, it is equally as likely that the benefits are because Plenadren causes reduced glucocorticoid exposure. The summary of product characteristics (SPC) advises using the identical dose of immediate-release hydrocortisone when patients switch to Plenadren, before individualising the dose (125). This practice was adopted in the aforementioned Plenadren studies. Comparing Plenadren once-daily to the equivalent thrice-daily immediate-release regimen, Plenadren causes a 6.4% increase in $AUC_{0\text{-}4h}$, but a further 30.5% and 58.8% decrease in $AUC_{4\text{-}10h}$ and $AUC_{10\text{-}24h}$ (101). The net effect is a 19.4% reduction in $AUC_{0\text{-}24h}$ and total glucocorticoid exposure. This reduction in glucocorticoid exposure would in turn explain why Plenadren is associated with worsening fatigue scores in SHS, and a tendency to increase dose on individualisation (104,106,126).

1.5.3 Diurnal Rhythmicity and CLOCK Genes

The rhythmicity of cortisol secretion has been well characterised and demonstrates a consistent pattern of acrophase on waking, followed by a decline in levels to a smaller lunchtime peak, dropping to an overnight basal level with nadir at approximately midnight (127-129). It has been equally well established that deviating from the physiological sleep-wake routine is associated with adverse mortality and morbidity outcomes (130). In a seminal publication, individuals in a papermill were followed up for over 15 years. Night shift worker demonstrated a significant increase in the relative risk of ischaemic events when compared to their daytime counterparts after 11 years, independent of other lifestyle factors.

Interrogation of medical examinations conducted on an Italian population of municipal workers over 44 years found that BMI, liver function tests and lipid profiles were elevated in individuals were on periods of night work (131). A recent metanalysis further quantified that shift work leads to a 17% increase in the rate of cardiovascular events, and after 5 years there is a subsequent 7.1% increase in events for every additional 5 years worked (132). Other studies have demonstrated an increased associated risk of diabetes, with an increased odds ratio of 2 for individuals who work at night (133). A randomised crossover study took healthy long-term shift workers and exposed to a simulated day-shift regimen and a simulated night-shift regimen for 3 days each, on two occasions (134). There was a 12-hour circadian misalignment achieved during the simulated night regimen, meaning that the participants were asleep between 11am and 7pm on the night regimen versus 11pm to 7am on the daytime regimen. Insulin and glucose responses to a standardised meal was recorded on both visits. It was noted that the night-shift protocol caused a 5.6% increase in postprandial glucose levels compared to the day-shift protocol. There was a concurrent 10% increase in late phase post-prandial insulin secretion, indicating that a circadian misalignment seen with night-shift patterns is associated with an increased insulin resistance.

The adverse cardiometabolic outcomes associated with night-shift work may be may be connected to the reverse cortisol profile that is observed in these individuals (135,136). There is evidence that the main apparatus which influences cellular timekeeping is the suprachiasmatic nucleus (SCN) of the hypothalamus (137). The SCN is unique in its direct innervation from the retinae, that allow for assessment of ambient light levels, which in turn can be used to ascertain the time of day. The SCN, serving as the "master clock", must then be able to convey this information to the "slave clocks" in the peripheral cells and tissues to maintain synchrony (138,139). One such mechanism by which this is achieved, is the HPA axis. Adrenal secretion of cortisol is temporally controlled by three pathways:

1- The local genetic timekeeping within the adrenal glands
2- Neuronal signalling from the SCN via the splanchnic nerves
3- ACTH secretion mediated by SCN influence

Cortisol secretion is in turn able to entrain the "slave clocks" of other peripheral cells and tissue to the correct time. The local cellular timekeeping in peripheral tissues is measured by oscillations in transcription and inhibition of "CLOCK genes" that are able to promote and inhibit the action of downstream messengers and actors depending on time of day and tissue type (140). The main loop, integral to the "CLOCK gene" system, involves circadian locomotor output cycle kaput (CLOCK) and its dimer, brain–muscle–arnt-like protein 1 (BMAL1). CLOCK/BMAL1 is transcribed and engages in feedback and transcriptional loops that involve enhancing further transcription of cryptochrome (CRY1-2) and period (PER1-3) genes (138). As CRY and PER accumulate and become phosphorylated, they autoregulate the loop by inhibiting transcription of CLOCK/BMAL1. As the CRYs and PERs are degraded and not being transcribed, the oscillation completes with the disinhibition of CLOCK/BMAL1, which again promote transcription of CRY and PER genes (Figure 1.3) (141). In addition to this main

loop, there are auxiliary loops which are able to inform the actions of other messengers and cellular responses.

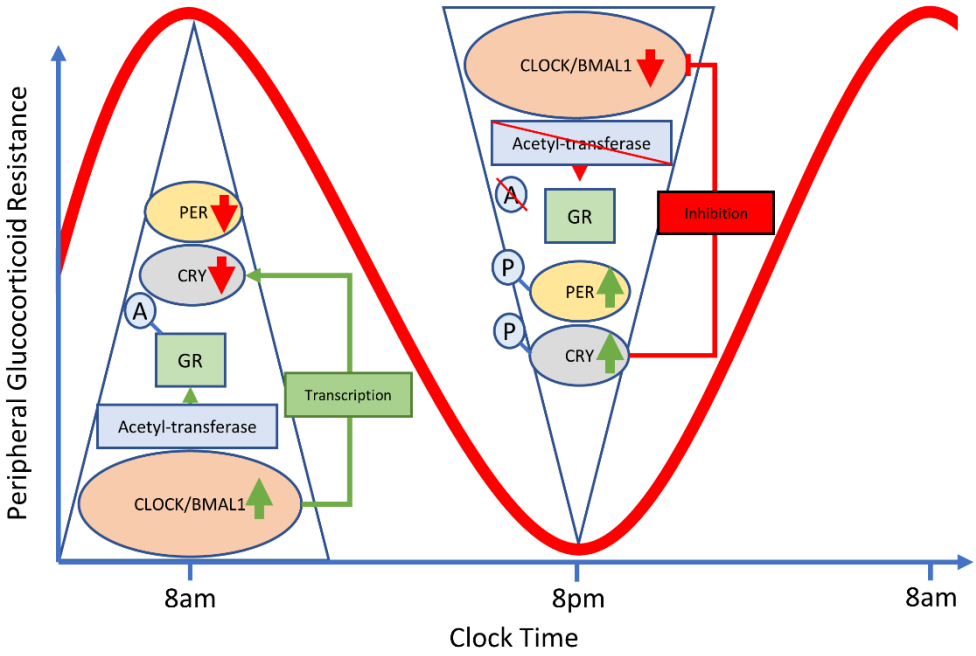

Figure 1.3- The CLOCK gene main loop and its effect on glucocorticoid resistance. At 8am, CLOCK/BMAL1 is highly expressed, which in turn acetylates (A) the cellular glucocorticoid receptors (GRs), causing attenuation and invoking resistance. CLOCK/BMAL1 also promotes expression of the PER1-3 and CRY1-2. In the evening, there is accumulation of phosphorylated (P) PER1-3 and CRY1-2, inhibiting CLOCK/BMAL1 and consequently inhibiting acetyltransferase activity. This leave GRs in a non-acetylated state, heightening sensitivity to glucocorticoids and reducing resistance.

1.5.4 CLOCK genes and cortisol

The GR is ubiquitously expressed by all cells with the exception of the SCN (142). This suggests that cortisol is able to influence timekeeping in all cells with the exception of the "master clock". It is however difficult to isolate human tissue with the intention of assessing the role of glucocorticoids in entraining the "slave clocks". One method is to isolate PBMCs as surrogate tissue. Sixteen healthy individuals were given either 20 mg hydrocortisone orally each day or placebo, 10 hours after waking for 6 days (143). PBMCs were collected regularly and CLOCK genes quantified and compared between groups and with individual baselines. The analysis revealed that single doses of hydrocortisone could cause significant increases in PER1 transcription after administration. Further, phase shifts were evident in some of the participants, whereby their peak PER2 levels occurred 9 hours after expected

and additional new peaks of PER3 in the evening were seen. This study demonstrates that a single dose of 20mg hydrocortisone can influence or “reset” the cellular timekeeping mechanisms.

There is evidence that glucocorticoid sensitivity also fluctuates during the day. Nine healthy males had their endogenous HPA axis pharmacologically suppressed and were given 50 mg of hydrocortisone at either 05:00 or 17:00, before their glycaemic axis was assessed (144). Regardless of the time of day, the cortisol pharmacokinetics were stereotyped. In the first 4 hours after the hydrocortisone administration, glucose handling and insulin secretion was unaffected by the time of day. However, when taken at 17:00, the peak glucose, and insulin secretion were significantly elevated between 4 and 16 hours after the hydrocortisone load compared to when it was administered at 05:00. This demonstrates greater sensitivity to glucocorticoids at 17:00 and lower sensitivity at 05:00.

It has been described that CLOCK/BMAL1 which is capable of acetylating the GR at its hinge, attenuating its function (138). With peak CLOCK/BMAL1 expression in the morning, the GR is in its most acylated state, prompting cortisol resistance. By evening, CLOCK/BMAL1 expression is low, leading to cortisol sensitivity. This has been demonstrated in PBMCs by comparing samples collected at 08:00 and 20:00 in 10 healthy volunteers showing a 2.8x increase in GR acetylation in the evening versus the mornings (Figure 1.3) (145).

Taken together, it is clear that there is a pattern of glucocorticoid sensitivity that follows an inverse diurnal rhythm compared to cortisol secretion. Specifically, when cortisol secretion peaks in the morning, cortisol sensitivity is low. Equally, when cortisol secretion is muted in the afternoon and evenings, cortisol sensitivity is high. Non-physiological patterns of cortisol secretion as seen in shift work, will therefore cause uncoupling of the “master clock” and the “peripheral clocks”, which can be entrained by cortisol. This dis-synchrony may be responsible for the adverse mortality and cardiovascular outcomes seen in the shift-work population. In addition to the uncharacterised effects

of cortisol secretion at non-physiological times, it is clear that the increased sensitivity to cortisol may offer a mechanism for developing diabetes, dyslipidaemia and hypertension. The clinical significance of this for AI patients is that glucocorticoid replacement regimens which predispose to having glucocorticoids in the blood at non-physiological times may contribute to the excess mortality. In thrice-daily hydrocortisone regimens, the late afternoon dose may cause glucocorticoids to be present in the system at the most detrimental time. Further, single doses of hydrocortisone may be sufficient to "reset" the clock at noon and in the afternoon, which may in turn further compound the uncoupling of the time keeping mechanisms. Conversely, once-daily regimens may protect against this.

1.5.5 CLOCK gene expression in Adrenal Insufficiency (AI)

The DREAM study has investigated the expression of CLOCK genes in the PBMCs of a selection of participants (146). This ancillary study included 26 patients who switched to Plenadren from standard hydrocortisone or cortisone acetate regimens, 29 patients who remained on their baseline standard hydrocortisone or cortisone acetate regimens and 16 healthy volunteers. CLOCK genes were quantified at baseline and at 12 weeks. At baseline, CLOCK/BMAL1 was downregulated in the AI cohort, with PER3 and TIMELESS (another CLOCK gene that is part of the PER negative feedback loop) were upregulated. Differences of expression were also noted in 16 other genes. Following 12 weeks of Plenadren there was normalisation of CLOCK/BMAL1, PER3 and TIMELESS expression to levels seen in the healthy volunteers. Of the remaining 16 genes, a further 15 had normalised.

These finding seem to confirm that conventional multiple-dose regimens of glucocorticoid replacement do change the expression of timekeeping genes in PBMCS. Whilst this does not prove that temporal dysregulation is the cause of the excess mortality in AI, it does provide further evidence that the benefits of a once-daily regimen may be mediated through reducing exposure at inappropriate times.

1.6 Future Approaches

Although there has been undeniable improvement in the care of patients with AI, there remains an unacceptable mortality gap which needs to be tackled. The three most prominent issues are currently:

1- The lack of an objective marker that can be used to gauge the adequacy of replacement regimens

2- The implied excess exposure resulting from the most common glucocorticoid regimens

3-The current risk of non-physiological replacement and nocturnal glucocorticoid exposure

1.6.1 Objective Markers of Replacement

In the absence of an objective marker to indicate the adequacy of replacement, it is difficult to ensure that a patient with AI is not being under- or over-replaced (147). In keeping with the usual practice in Endocrinology of monitoring the secondary hormone, ACTH is at first appealing. ACTH however shows marked ultradian pulsatility and may not reflect the subtle excess or deficit in a patient's regimen (148). Moreover, as ACTH responds directly to administered exogenous glucocorticoids, it is difficult to ascertain how soon to measure levels after a tablet is taken. There also may be issues if a patient inadvertently forgets to take their tablet on the day of quantification or does not absorb it fully. Its utility is further eroded in multidose regimens, where a level may be required multiple times in a day.

The DREAM study has suggested that immune cells may hold the key (108). PBMCs offer a surrogate "end organ" which may be useful to assess glucocorticoid exposure over preceding days or weeks. It is clear from the DREAM study that particular cell populations such as the non-classical monocytes and mature natural killer cell numbers may serve as an indicator of replacement status, but there are significant challenges. There are no current reference ranges for these cell populations and there is no evidence that a healthy reference range can be achieved by patients with adrenal insufficiency.

There may however be some utility in tracking these cell populations in an individual to assess relative adequacy of glucocorticoid replacement, much in the same way that trends of creatinine are indicative of relative renal function. There may also be scope to assess the CLOCK gene profiles in AI patients on replacement, to understand responses to individual doses, but the same aforementioned challenges exist. Further, the assays available for measuring specific cell populations and quantifying CLOCK gene mRNA expression are currently for research only with no specific platform designed for routine clinical care.

1.6.2 Glucocorticoid Excess in Current Regimens

Multidose hydrocortisone regimens remain the most popular treatment in the UK (65). The short hydrocortisone half-life mandates multiple doses per day, with peaks that exceed the height of physiological cortisol peaks and troughs that are deeper than physiological nadirs (69,149). The net effect of this "peak and trough" profile, is an AUC and subsequent glucocorticoid exposure that is higher than in healthy individuals. It is understood by clinicians that the "peak and trough" profile is a necessary evil, with the high peaks needed to mitigate against the low troughs and to reduce the risk of a crisis, even at the expense of slight over-replacement. It is however becoming apparent that the slight excess may be resulting in the significant increase in longer-term mortality and morbidity.

Once-daily medications may offer the solution to this problem. Both Plenadren and prednisolone both show a reduced AUC in comparison to thrice-daily hydrocortisone, whilst demonstrating sufficient control of the condition (90,99). The benefits of Plenadren in clinical trials has already been demonstrated (150). Although there is some evidence that low-dose prednisolone is no different to hydrocortisone in its effect on lipid profiles, there is a lack of studies comparing the two (151). This present study intends to address this vacuum of evidence.

1.6.3 Non-physiological and Nocturnal Glucocorticoid Exposure

It is not yet evident whether total glucocorticoid exposure or the level of non-physiological exposure is the greater cause of the excess mortality in AI. Multiple dose regimens of hydrocortisone do cause a cortisol profile that has three peaks which are not in keeping with the physiological cortisol profile (69). In addition, the evening dose of hydrocortisone predisposes to greater levels of cortisol in the blood at unphysiological times. Further compounding the issue, glucocorticoid sensitivity is heightened in the evening when glucocorticoid receptor acetylation is at its lowest (127,138). Once-daily preparation of glucocorticoids such as prednisolone and Plenadren, may protect against nocturnal dosing as glucocorticoid levels are typically low in the evening. Further they also offer daytime profiles that are closer to diurnal rhythm of cortisol, that can be achieved by thrice daily hydrocortisone (Figure 1.4) (90,101).

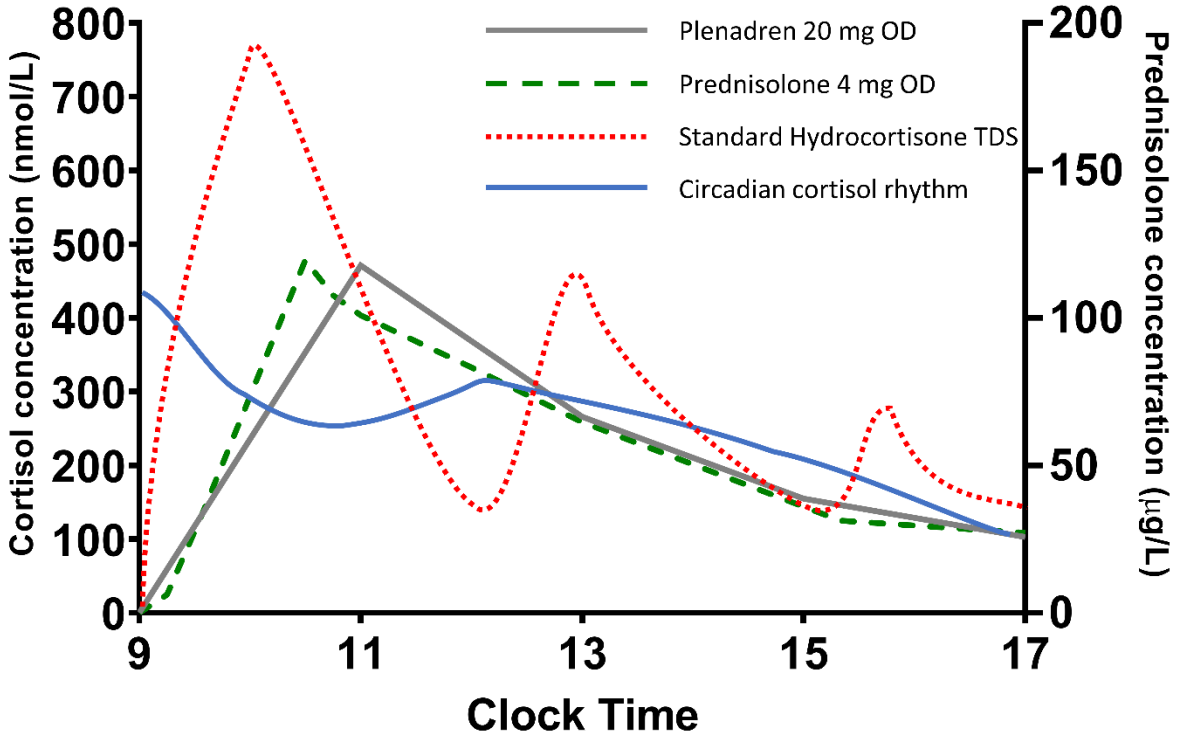

Figure 1.4- Comparison of the physiological cortisol rhythm and selected glucocorticoid replacement regimens. Circadian cortisol rhythm (solid blue) and standard hydrocortisone TDS (red dotted) have been plotted from data extracted from Mah et al 2004. Prednisolone curve (green dashed; n=1) and Plenadren (grey solid; n=1) curves are plotted from data collected at Imperial College Healthcare NHS Trust (ICHNT). The "peak and trough profile" of hydrocortisone can be seen to cause an excessive steroid exposure. Both prednisolone and Plenadren cause a profile that overshoots the cortisol profile to a lesser degree and is more physiological.

1.7 Study Aims

The aims of this thesis are to compare the effects of standard hydrocortisone therapy and low-dose prednisolone therapy for the management of AI, and anti-inflammatory high dose glucocorticoids on the indicators of:

1- Bone turnover

2- Cardiovascular risk

3- Glycaemic control

4- Infection rates

5- Immunological cell profiles

6- Wellbeing

1.8 Hypotheses

This thesis will test the following hypotheses:

1- Hydrocortisone and low-dose prednisolone therapy are equivalent in safety and efficacy

2- Novel indicators from routinely measured patient parameters can be used to indicate replacement status at a single timepoint

Chapter 2: Materials and Methods

2.1 Study Design and Subjects

The Objective Markers and New Indicators in Adrenal Insufficiency Disease (OMNI-AID Study) is a cross-sectional, observational pilot study. The study involves 4 specific groups:

- Group A: Healthy Volunteers
- Group B: Patients with AI who are managed with prednisolone therapy
- Group C: Patients with AI managed with hydrocortisone therapy
- Group D: Patients receiving high, anti-inflammatory doses of glucocorticoids to manage other medical conditions.

Up to 20 participants in each group were recruited between 22nd June 2018 and 26th March 2021. The study was conducted in the National Institute for Health Research (NIHR) Clinical Research Facility (CRF). Healthy volunteers were recruited from the healthy volunteer's database held by the CRF. Participants in Groups B and C were recruited from Endocrinology outpatient clinics held at ICHNT. Participants in Group D were recruited from Endocrinology outpatient clinics, or referred by the direct care teams based in Emergency departments and on the Planned Investigation Unit (where intravenous glucocorticoids are administered).

Participants were invited to attend up to two study visits. All participants were screened immediately prior to completion of study visit 1, and progressed to the study visit on the same day if they were eligible for the study and provided consent. At screening, a full medical history was taken, and a medical examination performed on all potential participants. Urinalysis was performed to exclude urine infections and pregnancy tests were completed on females with potential for pregnancy. Individuals in Group A were determined to be in good health with no significant medical conditions.

Individuals in Groups B-D, were assessed to ensure that they had been diagnosed with the relevant medical condition and were receiving glucocorticoid therapy as per the group definition. Second study visits were completed in individuals if the data collected in study visit 1 was compromised or if individuals in Groups B and C had changed from one glucocorticoid regimen to another as part of their routine clinical care. For the latter, the second study visit was completed at least 4 months after the participant had switched from prednisolone to hydrocortisone or vice versa. No maximum time period was specified.

All participants in this study provided written informed consent prior to being enrolled. This study was designed and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. Ethical approval for this study was obtained from the London-Stanmore Research Ethics Committee (reference number: 18/LO/0607).

2.2 Inclusion and Exclusion Criteria

The inclusion criteria for the OMNI-AID study mandate that participants (Groups A to D) are:

- Aged 18 – 85 years.
- Male or female.
- Otherwise, healthy enough to participate, as determined by pre-study medical history and physical examination.
- Able and willing to give written informed consent to participate in the study.

Patient groups required participants (Groups B to D) to be:

- Diagnosed with AI for over 6 months according to standard diagnostic criteria or with a medical condition requiring acute high dose glucocorticoid therapy for anti-inflammatory purposes.
- Established on stable HC replacement or prednisolone replacement (Groups B and C), dose not altered for at least 3 months.
- Established on a stable dose of Fludrocortisone (if PAI), if taking, dose not altered for at least 3 months.

- Stable on other hormone replacements (e.g. levothyroxine, testosterone or growth hormone in SAI), meaning that their replacement doses have not altered for at least 3 months.

The exclusion criteria for the study were as follows:

- Participants with a diagnosis of Type 1 or Type 2 diabetes mellitus (as glycaemic markers would be uninterpretable).
- Unable to give informed consent.
- Excessive caffeine intake above 500 mg per day.
- Taking supplements or herbal medications that the participant is unwilling or unable to stop prior to and during the study period e.g. St John's Wort (may decrease prednisolone levels), Cat's claw, Echinacea (immunomodulatory properties).
- Currently taking medications that alter CYP3A4 metabolism of glucocorticoids that the participant is unwilling or unable to stop prior to and during the study period e.g. phenytoin, phenobarbital, rifampicin, rifabutin, carbamazepine, primidone, aminogluethimide, itraconazole, ketoconazole, ciclosporin or ritonavir.
- Pregnancy, taking the oral contraceptive pill, or oral oestrogen replacement therapy due to the effects on cortisol binding globulin levels and determination of prednisolone levels. Transdermal oestrogen replacement is permitted. Females of child-bearing age will be asked to provide a urine sample for a pregnancy test at each visit.
- Growth hormone deficiency, if untreated.
- History of any medical, psychological or other condition, or use of any medications, including over-the-counter products, which, in the opinion of the investigators, would either interfere with the study.

2.3 Study Protocol

Participants in Groups A, B and C attended the CRF for study visits between 0700h and 1030h. Participants were instructed to fast from 2200h the night before and to avoid strenuous exercise and alcohol for 24 hours prior to their appointment. Participants in Groups B and C were instructed to administer their morning medication, in particular their glucocorticoid replacement therapy, at a fixed time to permit blood sample collection at 2 hours.

Participants in Group D either received oral or IV glucocorticoid treatment as per their routine clinical care. As the IV glucocorticoid administration usually took place in the afternoon, it was not practical to ask participants to fast prior to their study appointment. It was not possible to complete study visits on participants receiving IV treatment in the morning. Their blood samples were collected at 2 hours after the start of their IV infusion and urine samples were collected in the afternoon.

All study events were otherwise stereotyped (Figure 2.1). On attendance of their study visits, participants were asked to provide a urine sample (second sample of the day). Height was measured on the first occasion only. Body composition and weight was measured using a single Tanita body composition monitor located in the CRF. Waist and hip circumferences were measured using a method in accordance with the World Health Organisation (WHO) guidance (152). Participants were then asked to complete the study subjective health questionnaires, hosted on SurveyMonkey. Blood pressure and heart rate were recorded after the participant had been at rest during questionnaire completion. Where possible, blood samples were collected last of all, prior to discharging the patient. The described order of events was adhered to for the majority of visits. Deviations were made where a participant was unable to provide a urine sample immediately, or had mistimed glucocorticoid administration, whereupon blood samples were collected earlier than in the usual order.

Time *Illustrative*	**Screening Visit** *Combined with Visit 1 where possible*	**Visit 1**		**Visit 2** *(if completed)*	
(08:00)	---	Patient Groups only: Participant takes steroid tablets at home / receive IV steroids on ward	Minimum 1- week period	Patient Groups only: Participant takes steroid tablets at home / receive IV steroids on ward	Study Complete
09:00	Informed consent Medical history Physical examination Anthropometric measurements Urinalysis (±pregnancy test)	Informed consent		Informed consent	
09:15		Obtain urine sample (NTX ±pregnancy test)		Obtain urine sample (NTX ±pregnancy test)	
09:20		Anthropometric measurements collected		Anthropometric measurements collected	
09:30		Complete SF-36 and GNCQ questionnaires		Complete SF-36 and GNCQ questionnaires	
09:45		Blood pressure and heart rate recorded		Blood pressure and heart rate recorded	
10:00		Blood test Profile 1		Blood test Profile 1	

Figure 2.1- Schematic of study events to be completed at each study visit. Study visits are stereotyped and conducted in the morning (with practical exceptions made for Group D). NTX: N-terminal telopeptides; SF-36: Short Form health survey; GNCQ: German National Cohort Questionnaire; Blood profile 1:fasting renal, bone and lipid profiles, bicarbonate, full blood count (FBC), glucose, insulin, fructosamine, HbA1c, creatine kinase(CK), Adrenocorticotropic Hormone (ACTH), cortisol binding globulin (CBG), parathyroid hormone (PTH), vitamin D, osteocalcin (OC), procollagentype1 N-terminal propeptide(P1NP); hs-CRP, hs-Troponin I, BNP, peripheral blood mononuclear cells for white cell population analysis;

2.4 Study Outcomes

The primary outcome for this study is:

-Assessment of bone health

-Quantified by measurement of carboxylated osteocalcin (cOC) and undercarboxylated osteocalcin (uOC), which are bone formation and resorption markers respectively. Total OC will be derived from the sum of cOC and uOC. The uOC:cOC ratio (or OC ratio) will also be calculated.

The secondary and exploratory outcomes of this study are:

-Further assessment of bone health

-Quantification of supplementary bone turnover markers including serum procollagen type 1 N-terminal propeptide (P1NP) and urinary N-terminal telopeptide (NTX), a bone formation and resorption marker respectively. In addition, corrected calcium, parathyroid hormone (PTH) and Vitamin D will be measured.

-Risk factors for cardiovascular disease:

-Recording of vital statistics including blood pressure, heart rate, BMI, weight and waist-hip ratios (WHR). Mean arterial blood pressure (MAP) was calculated as ⅓SBP + ⅔DBP.

-Comparison of biochemical markers of risk including high sensitivity troponin I (hs-Trop), high sensitivity CRP (hs-CRP), brain natriuretic peptide (BNP), and lipid profiles.

-Glycaemic handling:

-Represented by long term markers such as HbA1c, shorter term markers such as fructosamine, and fasting glucose. Beta cell reserve will be estimated using homeostasis

model assessment of β-cell function (HOMA-%β) and insulin resistance will be estimated using homeostasis model assessment of insulin resistance (HOMA-IR).

-Infection rates:

-To be collected using the German national cohort questionnaire (GNCQ).

-Immunology profiles:

-To be derived from white cell populations characterised on full blood count (FBC) differentials. Further monocyte and NK cell populations will be characterised on flow cytometry and compared between groups.

-Subjective health:

-Measured using the short form health survey 36 (SF36) (153,154).

2.5 Sample Collection and Handling

2.5.1 Urinalysis

Urine samples were collected into white topped 30 ml universal containers. Urine dipsticks were performed using Siemens Multistix, and analysed using Siemens Clinitek Status+ analyser (Siemens Healthcare Limited, Frimley, UK). The urine samples were sent to North West London Pathology (NWLP) laboratory for quantification of NTX.

2.5.2 Blood sampling

Blood samples were collected in a single draw where possible using Vacutainer tubes (International Scientific Supplies Ltd, Bradford, UK). No more than 100 mls was collected into Becton Dickinson (BD) blood collection tubes (BD, Wokingham UK). In the following order of draw, 5 gold-top serum-

separating tubes, 3 red-top serum clot-activating tubes, 5 purple-top ethylenediaminetetraacetic acid (EDTA) coated tubes, 2 green-top lithium heparin coated tubes and 1 grey-top fluoride-oxalate tube were collected.

One purple-top, green-top and grey-top tubes were placed on ice immediately after collection. Two serum-separating tubes were allowed to clot at room temperature, typically taking approximately 5-10 minutes before being placed on ice. All tubes placed on ice, were immediately centrifuged at 4000 revolutions per minute (RPM) at 4°C to allow separation. Serum from one gold-top tube was separated into a new LP4 tube and sent to the NWLP laboratory on ice for quantification of insulin and c-peptide. The purple-top plasma, and grey-top plasma were also separated into separate LP4s and sent to the NWLP laboratory on ice for analysis of ACTH and glucose respectively. The remaining yellow-top serum, red-top serum and green-top plasma was stored at -80°C for subsequent OC, spare fructosamine and immunomarker analysis, respectively.

2.5.3 Peripheral blood mononuclear cells (PBMC) isolation

One further green-top tube was kept at room temperature prior to isolation of PBMCs. Within 4 hours of sample collection, 6 mls of neat whole blood was pipetted into sterile prepared Leucosep tubes (Greiner Bio-one, Gloucestershire, UK) containing 3 mls of Ficoll-Paque Plus (Sigma, Poole, UK) and 3mls of pre-warmed RPMI (Sigma, Poole, UK). The Leucosep tube was then centrifuged at 1200g for 15 minutes at room temperature. The buffy coat was then carefully removed using a wide aperture sterile pipette, to a 50 ml Falcon tube (Fisher Scientific, Loughborough, UK) containing 40 mls of warmed complete RPMI (cRPMI). cRPMI is a preprepared solution of RPMI, containing 10% foetal bovine serum (FBS) (Sigma, Poole, UK) and 1% antibiotic/antimycotic solution (Sigma, Poole, UK). The Falcon tube was centrifuged at room temperature for 7 minutes at 250g. The supernatant was subsequently poured of the newly formed pellet and the pellet of cells was resuspended in 2 mls of

12.5% bovine serum albumin (BSA) (Sigma, Poole, UK), which has previously been diluted with RPMI. Freezing medium at a concentration of 5% BSA, and 20% dimethylsulfoxide (DMSO) (Sigma, Poole, UK) in RPMI, was then added dropwise to the suspended PBMCs. The final solution was aliquoted into cryovials (Alpha Labs, Hampshire, UK), before being cooled slowly to -80°C in a Mr Frosty container (Fisher Scientific, Loughborough, UK). The aliquots were then stored long-term at -80°C prior to analysis by flow cytometry. The above procedures were completed in a sterile environment maintaining in a Class 2 bio-safety cabinet.

2.6 Assay Methodology

2.6.1 Osteocalcin quantification

Osteocalcin, both cOC and uOC were both measured using commercial enzyme linked immunosorbent assays (ELISAs) (Takara Bio, Saint-Germain-en-Laye, France). The cOC assay demonstrated a lower limit of quantification (LLoQ) of 0.5 ng/ml, with a reported inter- and intra- assay coefficient of variation (CV) of <2.4% and <4.8% respectively. The uOC assay has a LLOQ of 0.25 ng/ml with inter- and intra-assay imprecision of <6.7% and <9.9% respectively.

2.6.2 Flow cytometry

Frozen PBMCs were thawed to 37°C in an incubator for 10 minutes and resuspended in 10 ml of warmed RPMI. They were washed in 10 mls of phosphate buffered saline (PBS) (Sigma, Poole, UK). After centrifuging for 7 minutes at 400RCF, the pellet was reconstituted at a target concentration of 10 x10^6 PBMC/ml. The cells were then incubated with 2.5 µg of human Fc block (BD, Wokingham, UK) for 10 minutes prior to staining with antibodies in Table 2.1. All antibodies were purchased from BD, Wokingham, UK. Cells were incubated with antibody mixes for 30 minutes in the dark at room temperature. They were washed in 2 ml of PBS and finally resuspended in 400 µL of PBS with 0.5% paraformaldehyde.

Acquisition was completed on a BD LSR II using DIVA software (BD, Wokingham, UK). A minimum of 50,000 events was recorded for each sample. Compensation was done using control unstained cells and Versa Comp beads (Beckman Coulter, Wycombe, UK). Analysis was completed using FlowJo 10.8.1 (BD, Wokingham UK). Gating strategies for monocytes and NK cells are outlined in Figures 2. 2 and 2.3 respectively.

	Fluorochrome					
	FITC or Alexa 525/50 B.A.	**BV421** 450/50 V.A.	**V500** 525/50 V.A.	**BV786** 780/60 V.A.	**PE** 575/26 YG.A	**APC or Alexa Fluor 647** 660/20 R.A.
Monocyte panel	HLA-DR	CD206	CD14	CD74	CD16	CD86
NK cell panel	CD56	CD45	CD3	NKp46	CD16	NKG2D

Table 2.1- Antibody panel used for flow cytometric detection of monocytes and natural killer (NK) cells.

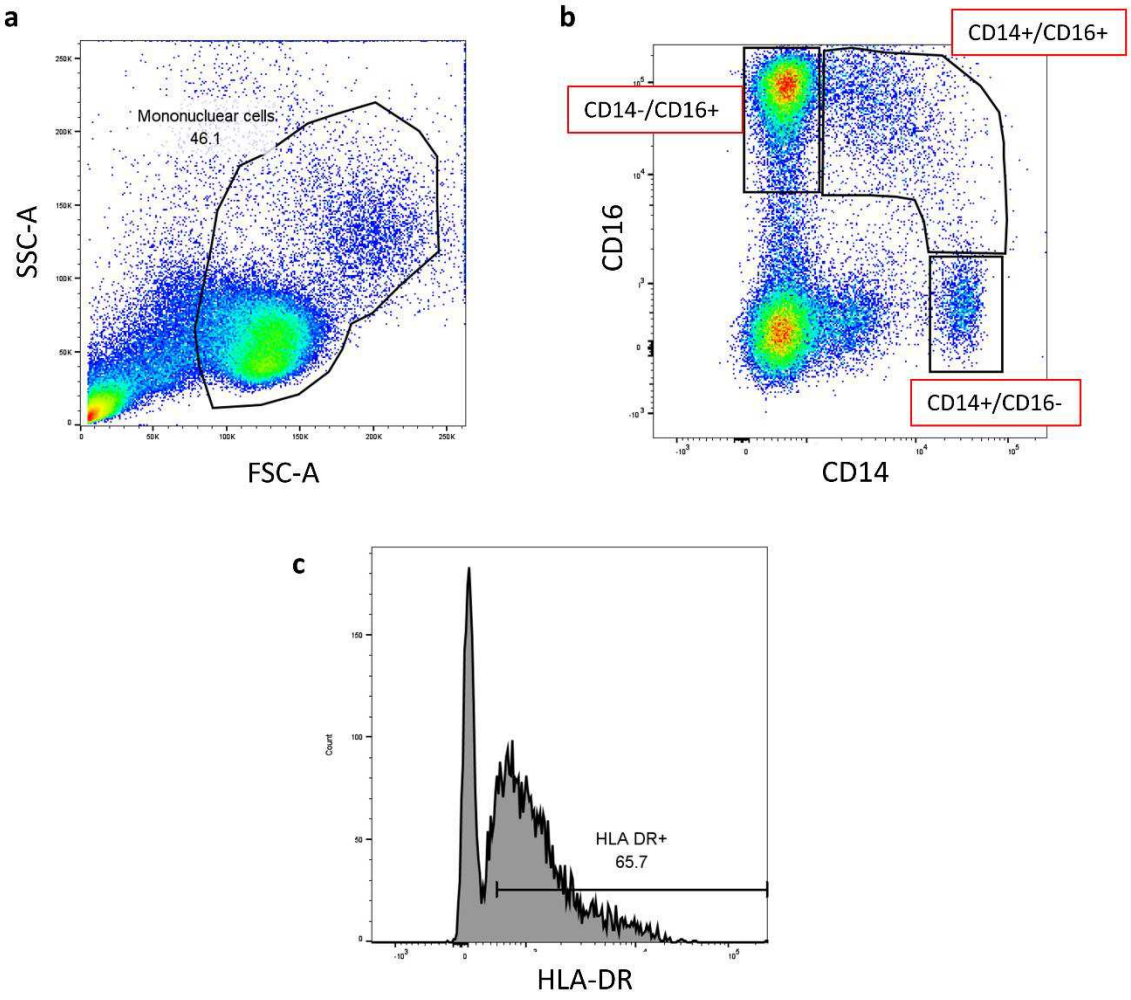

Figure 2.2- Gating strategy for Monocytes. Mononuclear cells were identified on forward versus side scatter **(**a). CD14+/CD16- classical monocytes, CD14+/CD16+ intermediate and CD14-/CD16+ non-classical cells were gated on in the waterfall plot (b). HLA-DR expression on all monocytes was identified on a histogram using gates set from unstained controls(c).

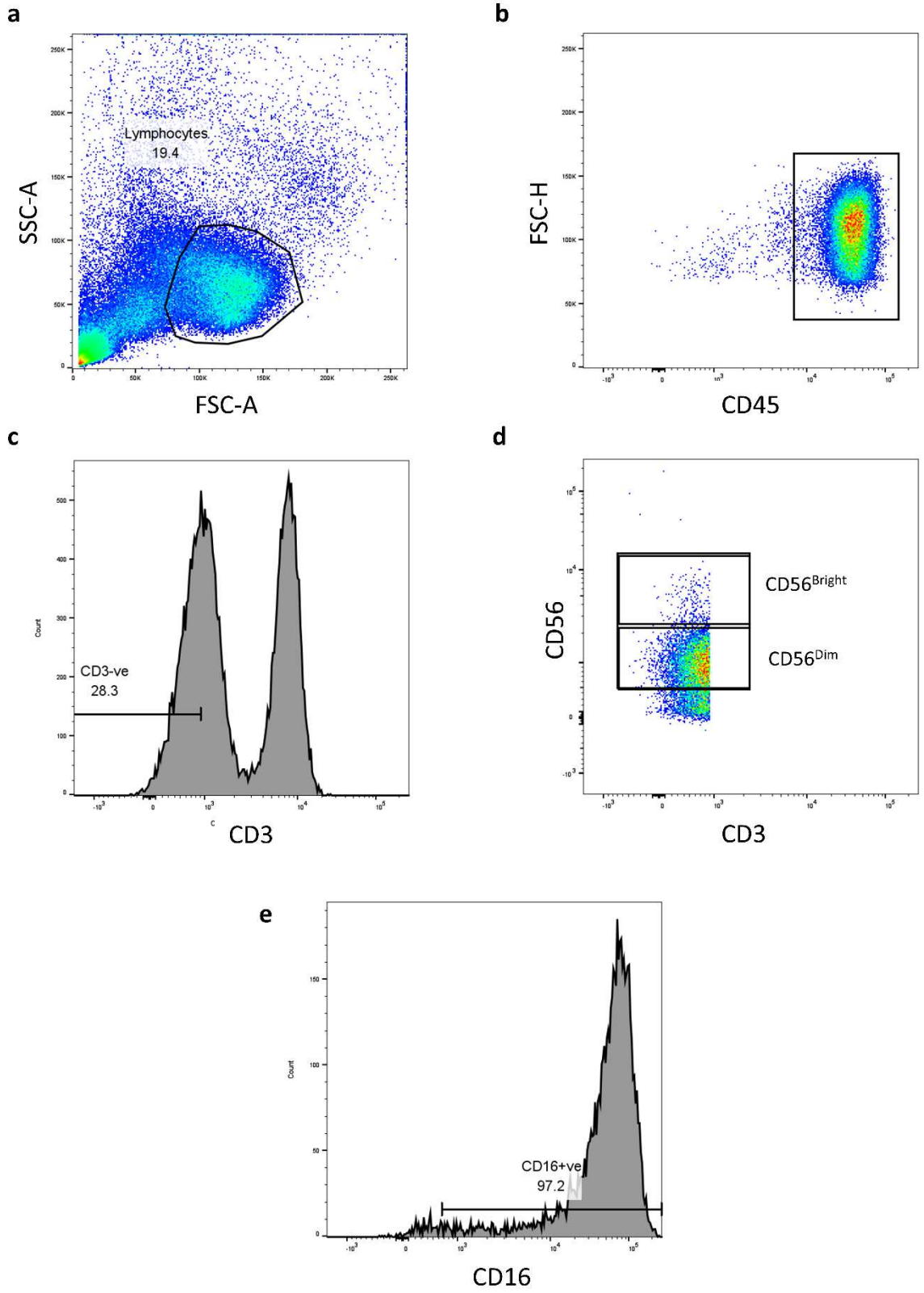

Figure 2.3- Gating strategy for natural killer (NK) cells. Lymphocytes were identified on forward vs side scatter (a). CD45+ cells were gated (b). CD3- gate on histogram was identified using unstained controls. (c). CD56Bright and CD56Dim cells were gated(d). CD56+/CD16+ cells were identified from the histogram (e).

2.6.3 Other analyte analysis

All other analytes were measured by pathology services at NWLP. All specimens were collected in keeping with sample collection requirements published by NWLP, unless otherwise specified as in the case of urine NTX in Group D participants. The majority of analyte quantification was performed on the Abbott Architect platform, with few samples analysed on the Abbott Alinity platform. NWLP undertook a phased transition from the Architect platform to the Alinity between 2018 and 2019. As most of the analysis was done on Architects, the Architect data has been provided unless otherwise stated. During the transition, the Alinity analysers were directly validated against Architects, with no changes in any of the reference ranges. The assay platforms used and performance specification are outlined in Table 2.2.

Assay	**Platform**	**Lower Limit of Quantification (LLOQ)**	**Inter-assay CV**	**Intra-assay CV**
P1NP	Roche Cobas	5 ng/ml	<3.7%	<3.2%
Urine NTX	Commercial Osteomark ELISA	1 BCE nmol/L	<5.0%	<19.0%
Creatinine (urine)	Abbott Architect	0.442 mmol/L	<2.0%	<1.7%
Calcium	Abbott Architect	0.25 mmol/L	<1.0%	<0.6%
Albumin	Abbott Architect	3.1 g/L	<1.2%	<0.4%
PTH	Abbott Architect	0.3 pmol/L	<8.7% (Total CV)	<8.7%
Vitamin D	Abbott Architect	8.5 mmol/L	<7.1%	<5.1%
Creatinine (serum)	Abbott Architect	17.7 µmol/L	<1.9%	<1.5%
Potassium	Abbott Architect	1.0 mmol/L	<1.2%	<0.7%
Bicarbonate	Abbott Alinity	4 mmol/L	<3.9%	<2.2%
hs-Trop	Abbott Alinity	2 ng/L	<7.2%	<5.0%
hs-CRP	Abbott Architect	0.1 mg/L	<4.00%	<2.38%
BNP	Abbott Architect	10 ng/L	<6.7% (Total CV)	<5.6%
HbA1c	Tosoh G8	3 mmol/mol (<20mmol/mol not routinely reported)	<2.6% (Total CV)	<2.4%
Fructosamine	City Assays- In house colorimetric assay	10 mmol/L	<2.5%	<1.0%
HDL	Abbott Alinity	0.13 mmol/L	<5.1%	<1.7%
Triglycerides	Abbott Architect	0.071 mmol/L	<1.7%	<0.8%
Cholesterol	Abbott Architect	0.18 mmol/L	<1.4%	<1.1%
Glucose	Abbott Architect	0.278 mmol/L	<0.99%	<1.98%

Insulin	Abbott Architect	1.0 mIU/L	<5.2%	<4.2%
C-peptide	Abbott Architect	3.31 pmol/L	<4.0% (Total CV)	<2.4%
FBC	Sysmex XE2100	N/A	N/A	N/A
ACTH	Siemens Immulite	5 ng/L	<10.0%	<9.5%
Cortisol	Abbott Architect	28 nmol/L	<6.2%	<5.5%
Prednisolone	In-house HPLC-MS/MS	10 µg/L	<6.5%	<6.5%

Table 2.2- Assay platform and performance specification for analytes measured at North West London Pathology (NWLP). C-peptide, parathyroid hormone (PTH), brain natriuretic peptide (BNP) and HbA1c inter-assay imprecisions were not reported. The total imprecision has been stated instead. Architect data was not available for bicarbonate, high sensitivity troponin I (hs-Trop) and high density lipoprotein (HDL). Alinity data has been provided instead.

2.7 Statistical Analysis

As this is a pilot study, a formal power calculation could not be performed. In this observational cross-sectional study, the distribution of each individual dataset was initially assessed using the Shapiro-Wilk test. Parametric data between groups was analysed using a one-way ANOVA and Tukey's test was utilised for post-hoc multiple comparison. Non-parametric data was analysed with a Kruskal-Wallis test, and subsequent Dunn's Test. Some participants in Groups B and C may have attended 2 study visits after switching between prednisolone and hydrocortisone therapy. This data was separately analysed. For parametric datasets, a paired t-test or repeated measures ANOVA was used. For non-parametric data, analysis was completed using a Wilcoxon test. For correlation analysis, a scatterplot was constructed, and Pearson's correlation coefficient was calculated and reported. Best fit line were drawn using ordinary least squares regression. The Chi-squared test was employed to assess categorical data. Statistical significance was defined as $P<0.05$.

Chapter 3: The Effects of Different Glucocorticoid Regimens on Bone Turnover

3.1 Introduction

Glucocorticoids have been long described to cause osteoporosis and increase the risk of femoral and vertebral fractures due to glucocorticoid induced osteoporosis (GIO) (155). Cumulative exposure to glucocorticoids is strongly correlated with BMD loss. Doses in excess of prednisolone 5 mg daily for greater than 3 months is known to be detrimental with the effect of doses greater than 7.5 mg, being even more profound (156,157). Glucocorticoid therapy induces bone loss by multiple mechanisms (158,159). There is direct inhibition of osteblastogenesis, in addition to inducing apoptosis in mature osteoblasts and osteocytes (160,161). Further, Dickkopf-1 (DKK1) mediated inhibition of canonical Wnt signalling directly attenuates mature osteoblast function (162,163). In the acute setting, the lifespan of osteoclasts is noted to be disproportionately increased, uncoupling bone formation and bone resorption rates (164). This is in keeping with observations that glucocorticoid exposure reduces osteoprotegerin (OPG) secretion by osteoblasts, increases receptor activator of nuclear factor κB ligand (RANK-L) expression and augments macrophage colony stimulating factor (MCSF), all of which promote osteoclastogenesis (165,166). As a result of these effects, GIO ostensibly affects the trabecular bone of the vertebrae.

The best approach to investigate the effects of different glucocorticoid regimens on bone turnover, would involve quantitative CT scanning or DEXA imaging to estimate BMD. Intervals between scans are usually more than 2 years, meaning that imaging studies are longer term and would require an extended period of enrolment for the participant (167). As surrogate marker of BMD change, biochemical markers of bone turnover can be measured in the shorter term.

Bone turnover markers are separated into bone formation markers and bone resorption markers. The formation markers include total OC and P1NP. The main resorption marker utilised in this study is urinary NTX.

Total OC, the sum of cOC and uOC, has been long associated with osteogenesis (168). It is a 49-amino acid protein that undergoes vitamin-K dependent carboxylation of three glutamine residues in osteoblasts (169). This permits the protein to bind calcium and hydroxyapatite molecules, and as such, it is the most abundant non-collagenous protein in the bone extracellular matrix. OC is an attractive bone marker to quantify in a short-term study due to its ability to demonstrate rapid change in response to oral and inhaled corticosteroids after 7 days (170). P1NP is the N-terminal fragment of procollagen after it has been cleaved to form collagen I, an essential scaffold protein of the bone matrix (171). P1NP is stable at room temperature and intra-individual variation of P1NP levels have been characterised and is noted to be low (172,173). P1NP has demonstrated a change from baseline in response to osteoporosis treatment, as early as 4 weeks after the treatment has started and settles on a new baseline after 3-6 months of treatment (174).

Urinary NTX is a breakdown product of collagen 1, when it is cleaved by cathepsin-K (175). NTX has also demonstrated a response to bone anti-resorptive therapy at 4 weeks, reaching a baseline between 3 and 6 months (174). uOC has previously been thought of as another marker of osteoclastic activity following observations that elevated levels were associated with increased risk of hip fracture in elderly women (176). It is becoming increasingly apparent that uOC functions as a hormone, indicating the status of energy metabolism of the bone (177). Further, there is evidence that uOC may in fact be secreted by osteoblasts when gamma-carboxylase, which is responsible for carboxylating the glutamine residues, is inhibited (178). Although the role of uOC has not been fully elucidated, it has been measured as a bone marker in this study.

The relationship between biochemical bone marker and BMD has been characterised in pathological states, particularly osteoporosis (179). In a study of 723 men who were followed up over 7.5 years with DEXA scans and biochemical bone turnover markers measured every 18 months, elevated levels of biochemical markers were associated with BMD loss (180). C-terminal telopeptide (CTX), which is analogous to urinary NTX, and OC elevations showed a positive correlation with BMD reduction at the distal radius and ulnar. P1NP in particular, has been investigated in post-menopausal women with osteoporosis (181). Compared to healthy pre-menopausal women, they demonstrated 74% higher levels of P1NP, indicating that elevated levels are associated with lower BMD.

In contrast to the above understanding of bone turnover markers in the context of healthy individuals and individuals with osteoporosis, there is evidence that increasing exposure to glucocorticoids causes suppression of bone formation markers and causes an indeterminate effect on resorption markers (182). Therefore, bone marker assessment in glucocorticoid replacement studies considers increases in bone formation markers as a positive outcome, indicative of lower glucocorticoid exposure as opposed to indicating an increased risk for osteoporosis and fractures. Thirty-two healthy males were allocated to either prednisolone 7.5 mg once-daily, prednisolone 30 mg once-daily or placebo for two weeks. P1NP, OC and CTX were quantified at baseline and after the period of intervention. Dose-dependent reductions from baseline in OC and P1NP were noted in the prednisolone 7.5 mg and 30 mg groups. These reductions were significant when compared to the placebo group. No significant changes were noted in the CTX levels.

Another study recruited 20 healthy volunteers, 19 patients with untreated Cushing's syndrome, 16 patients with AI receiving a median of 20 mg hydrocortisone equivalent replacement in the form of hydrocortisone or cortisone, and 13 patients on chronic prednisolone treatment receiving a median of 14mg daily for a median of 24 months (183). OC levels were measured at 3 timepoints during the day, and remained stable in the healthy volunteers. They were significantly suppressed by

administration of dexamethasone 4 mg in the healthy volunteers. The Cushing's cohort and the chronic prednisolone group were both noted to have suppressed OC levels compared to the healthy volunteer and AI group. OC levels between the healthy volunteers and AI patients were similar.

There is sufficient evidence of bone formation marker suppression during glucocorticoid excess, but there remains a void of evidence concerning bone marker levels in glucocorticoid deficient adults. This is because patients are never knowingly under-replaced and there is no reason to measure bone markers in an acute setting. Suppression of bone markers can therefore characterise glucocorticoid over-replacement but are ambiguous in under-replaced states. Statistically significant relative differences in bone marker levels between treatments can still highlight suppression due to excess glucocorticoids in a replacement regimen given the dose-dependent relationship. Bone markers are therefore measured between all 4 groups in the present study.

3.2 Hypotheses and aims

3.2.1 Hypotheses

1- Bone formation markers will be comparable between patients receiving hydrocortisone replacement and prednisolone replacement therapy and equivalent to levels in the healthy volunteers
2- NTX will be the same between all groups
3- Patients receiving high doses of glucocorticoids will show suppressed formation and resorptive markers

3.2.2 Aims

To compare bone formation and resorption markers between patients taking hydrocortisone and prednisolone replacement regimens in AI, as well as with healthy volunteers and patient receiving high doses of glucocorticoids.

3.3 Results

3.3.1 Baseline demographic data

Twenty healthy volunteers, 20 AI patients receiving prednisolone, 20 AI patients receiving hydrocortisone and 9 patients receiving high dose glucocorticoids were recruited to this study. Table 3.1 shows their baseline characteristics. Within Group D, 3 individuals with Graves' eye disease were treated from ophthalmopathy, and 5 for orbitopathy. One individual with allergic reaction, and another with ophthalmopathy were treated with prednisolone, all others received IV methyl-prednisolone (IVMP).

Characteristics	**Group A** **Healthy Volunteers**	**Group B** **Prednisolone**	**Group C** **Hydrocortisone**	**Group D** **High Dose**
N number	20	20	20	9
Age (years)	60.0 (34.3)	58.5 (22.0)	63.0 (12.3)	55.0 (18.0)
Gender	10F 10M	12F 8M	9F 11M	8F 1M
Weight (kg)	71.4 (13.2)	67.7 (11.9)	75.7 (24.0)	63.5 (10.9)
Type of AI / condition	Not applicable	6x 1° 14x 2°	8x 1° 12x 2°	8x Graves' eye disease 1x Allergic reaction
BMI (kg/m^2)	24.2 (2.8)	23.9 (5.4)	25.5 (6.8)	23.3 (4.8)
Dose (mg)	Not applicable	3.3 (±0.7)	20.8 (±6.7)	2x 1000 mg IVMP 5x 500 mg IVMP 1x 60 mg prednisolone 1x 40 mg prednisolone

Table 3.1- Baseline characteristics of participants in the study. Age, weight and body mass index (BMI) are reported as median (interquartile range (IQR)). Prednisolone and hydrocortisone doses are reported as mean (±SD). IVMP- IV methyl-prednisolone.

3.3.2 Osteocalcin data

Figure 3.1 shows the differences in levels of OC and the OC ratio between groups. The participants receiving high dose glucocorticoids in Group D have a lower median (IQR) uOC of 2.37 (2.83) ng/ml, and mean (±SD) cOC of 12.94 (±4.86) ng/ml compared to the healthy volunteers in Group A who have corresponding values of 6.78 (4.26) ng/ml and 23.72 (±10.2) ng/ml respectively. This did not reach significance. The AI treatment groups were comparable with a median uOC and mean cOC of 6.43 (6.79) ng/ml and 21.69 (±7.13) ng/ml in the prednisolone group and 5.60 (4.51) ng/ml and 27.09 (±14.45) ng/ml in the hydrocortisone group.

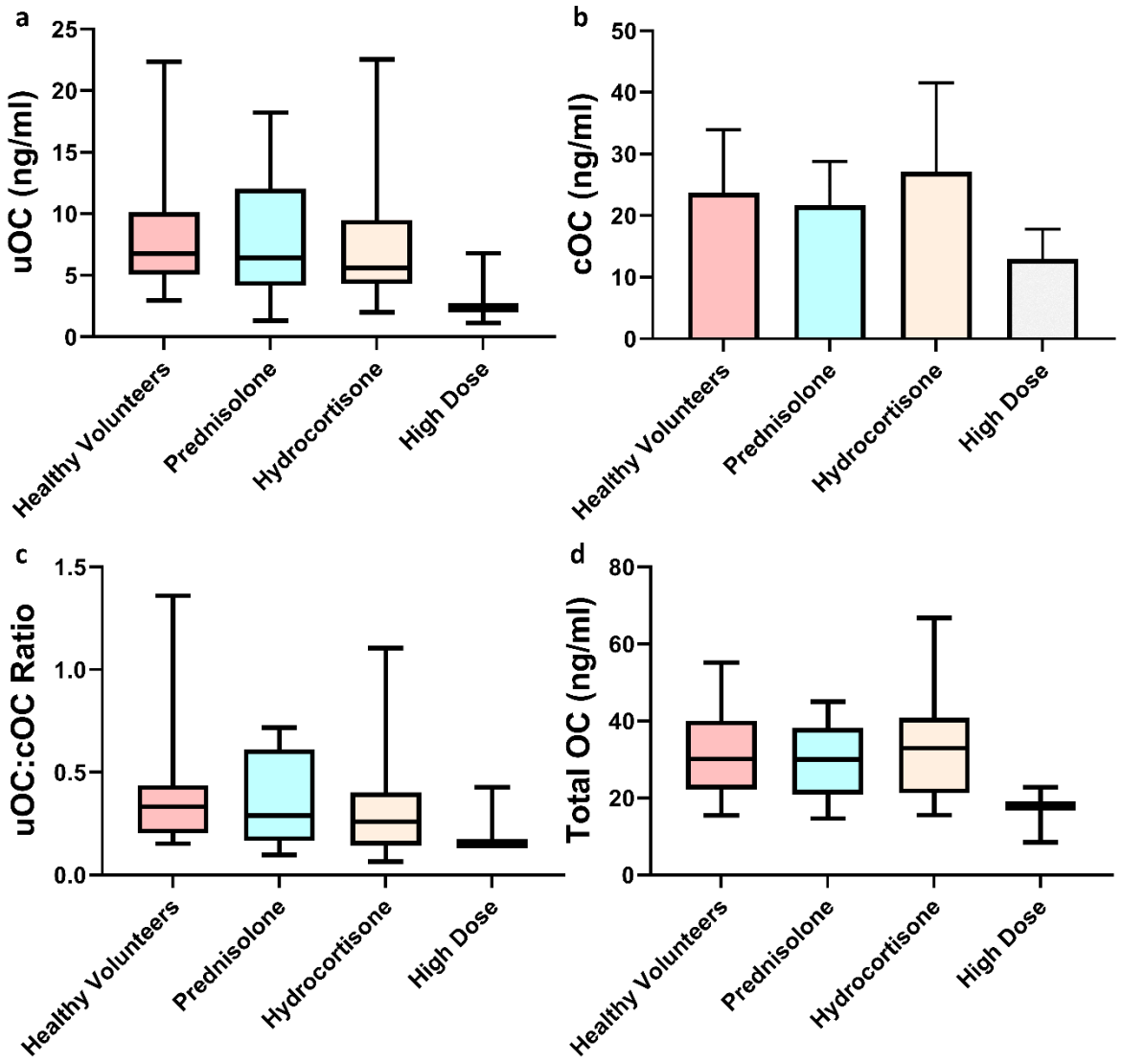

Figure 3.1- Differences between groups in undercarboxylated osteocalcin (uOC) (a), carboxylated osteocalcin (cOC) (b), uOC to cOC ratio (c) and total OC (d). Data is given as median, quartiles and range in (a), (c) and (d). Data is given as mean + SD in (b). Healthy volunteers n=20; prednisolone n=18; hydrocortisone n=16; high dose n=7. No significant differences between groups were seen.

Further assessment of Groups B and C, showed that increasing prednisolone dose trended with decreasing uOC levels, although this did not reach significance (Figure 3.2). In keeping with the observed trend, a significant negative correlation was formed between the prednisolone dose and OC ratio, which is a function of uOC. Increasing hydrocortisone dose was conversely associated with significant decreases in cOC and total OC (Figure 3.3).

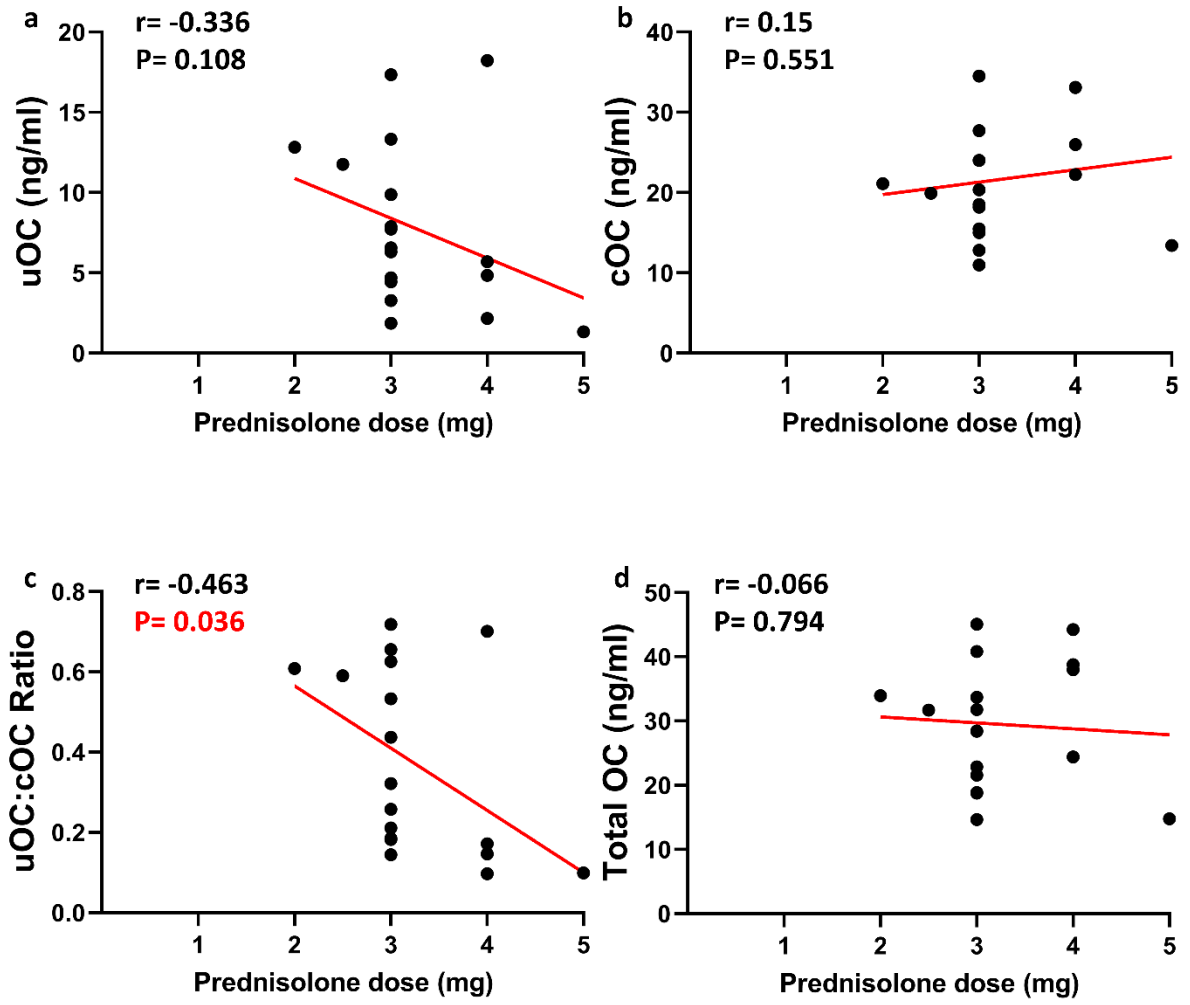

Figure 3.2- Scatterplot of Prednisolone dose versus undercarboxylated osteocalcin (uOC) (a), carboxylated osteocalcin (cOC) (b), uOC to cOC ratio (c) and total OC (d). Least squares regression line fitted. Pearson's correlation coefficient and P-values are reported; n=18. A significant moderate correlation was observed between prednisolone and OC ratio (c).

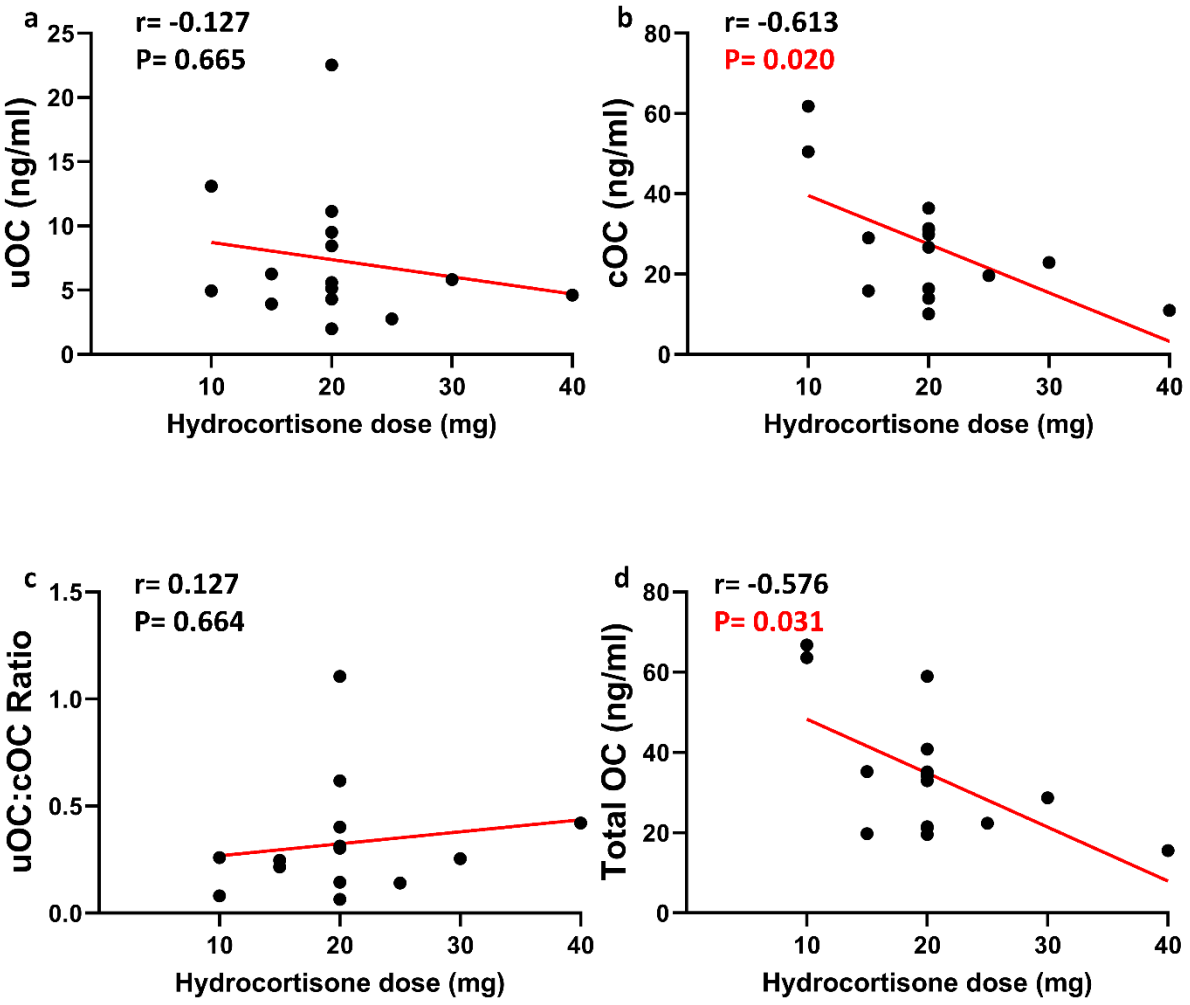

Figure 3.3- Scatterplot of Hydrocortisone dose versus undercarboxylated osteocalcin (uOC) (a), carboxylated osteocalcin (cOC) (b), uOC to cOC ratio (c) and total OC (d). Least squares regression line fitted. Pearson's correlation coefficient and P-values are reported; n=16. A significant moderate negative correlation was observed between hydrocortisone and cOC (b) and total OC (d).

3.3.3 Further bone marker and biochemical data

Median (IQR) P1NPs were 52.2 (30.9) ng/ml, 39.0 (32.9) ng/ml, 41.0 (20.3) ng/ml, 49.0 (19.1) ng/ml for Groups A-D respectively. Urinary NTX levels were also comparable between all groups with no significant differences noted (Figure 3.4).

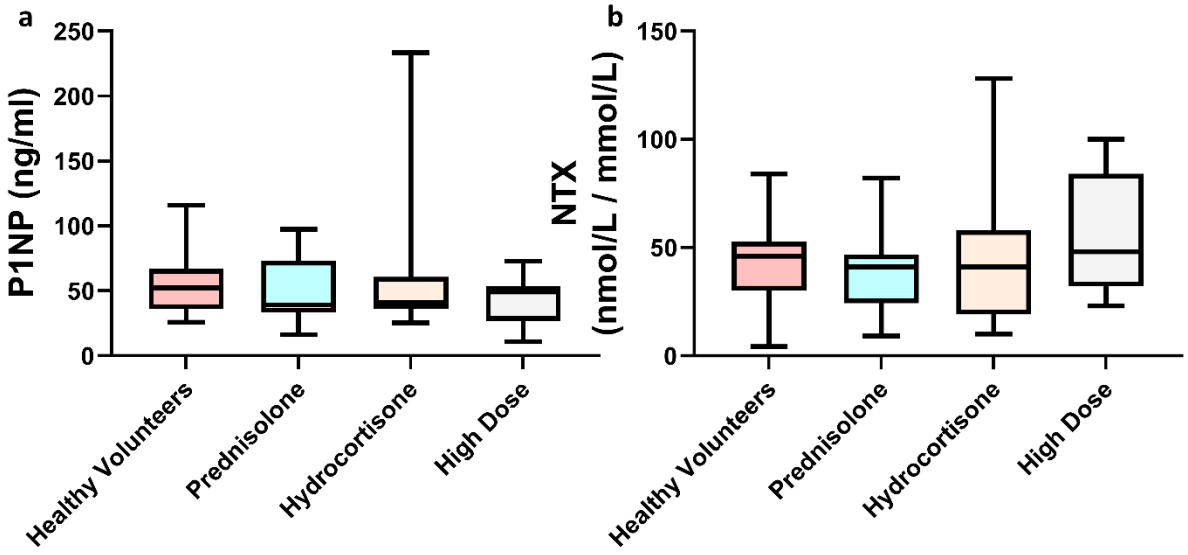

Figure 3.4- Differences between groups in procollagen type 1 N-terminal propeptide (P1NP) (a) and N-terminal telopeptide (NTX) (b). Data is given as median, quartiles and range. Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=19; high dose n=9. No significant differences between groups were seen.

Corrected calcium levels, phosphate levels and vitamin D were similar across the groups (Table 3.2). PTH levels were however significantly elevated in AI patients taking hydrocortisone in Group C at 8.2 (±3.2) pmol/L versus 6.1 (±2.3) pmol/L in Group A; P= 0.04 (Figure 3.5).

Parameter	**Group A** **Healthy Volunteers**	**Group B** **Prednisolone**	**Group C** **Hydrocortisone**	**Group D** **High Dose**	**P-value (ANOVA/Kruskal Wallis)**
Corrected Calcium (mmol/L)	2.35 (±0.09)	2.39 (±0.09)	2.39 (±0.07)	2.36 (±0.11)	0.40
Phosphate (mmol/L)	1.11 (0.19)	1.12 (0.29)	1.02 (0.12)	1.05 (0.20)	0.22
Vitamin D (nmol/L)	52.8 (30.2)	82.3 (32.4)	85.1 (35.1)	70.1 (42.3)	0.49
PTH (pmol/L)	6.1 (±2.3)	6.4 (±2.2)	8.2 (±3.2)	6.5 (±1.9)	*0.04

Table 3.2-Results of measured biochemical bone markers. Data is presented as mean (±SD) for parametric data, and median (IQR) for non-parametric. Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=19; high dose n=9. * denotes significant P-values.

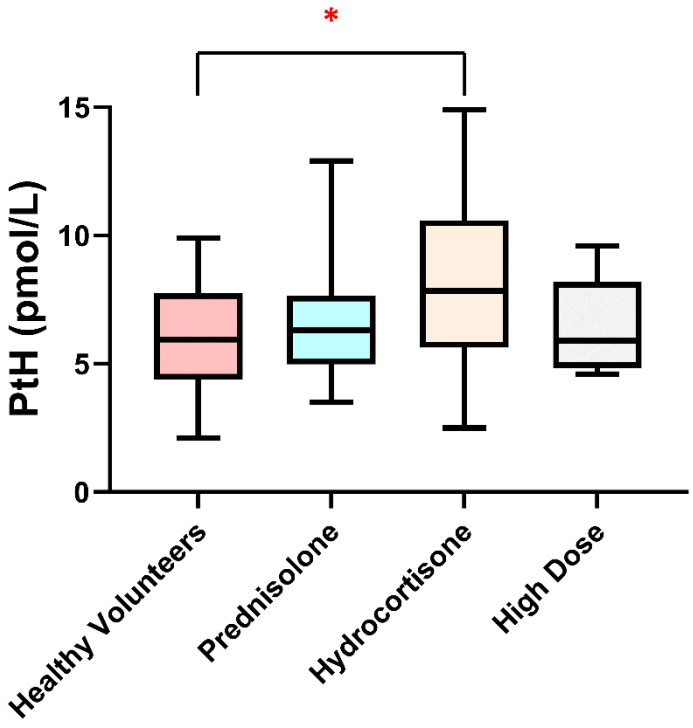

Figure 3.5- Differences between groups in parathyroid hormone (PTH). Data is given as median, quartiles and range. Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=19; high dose n=9. *denotes P=0.04

3.3.4 Data from crossover analysis

Five individuals with AI in Groups B and C had data for uOC and cOC whilst receiving both prednisolone and hydrocortisone replacement therapy. A further 4 individuals, making a total of 9, had additional data for P1NP, NTX, PTH, corrected calcium and phosphate (Figure 3.6). No significant differences were detected in these parameters between both treatments. PTH levels were noted be higher in 6 individuals when they were taking hydrocortisone as opposed to prednisolone, with three noted to demonstrate the converse.

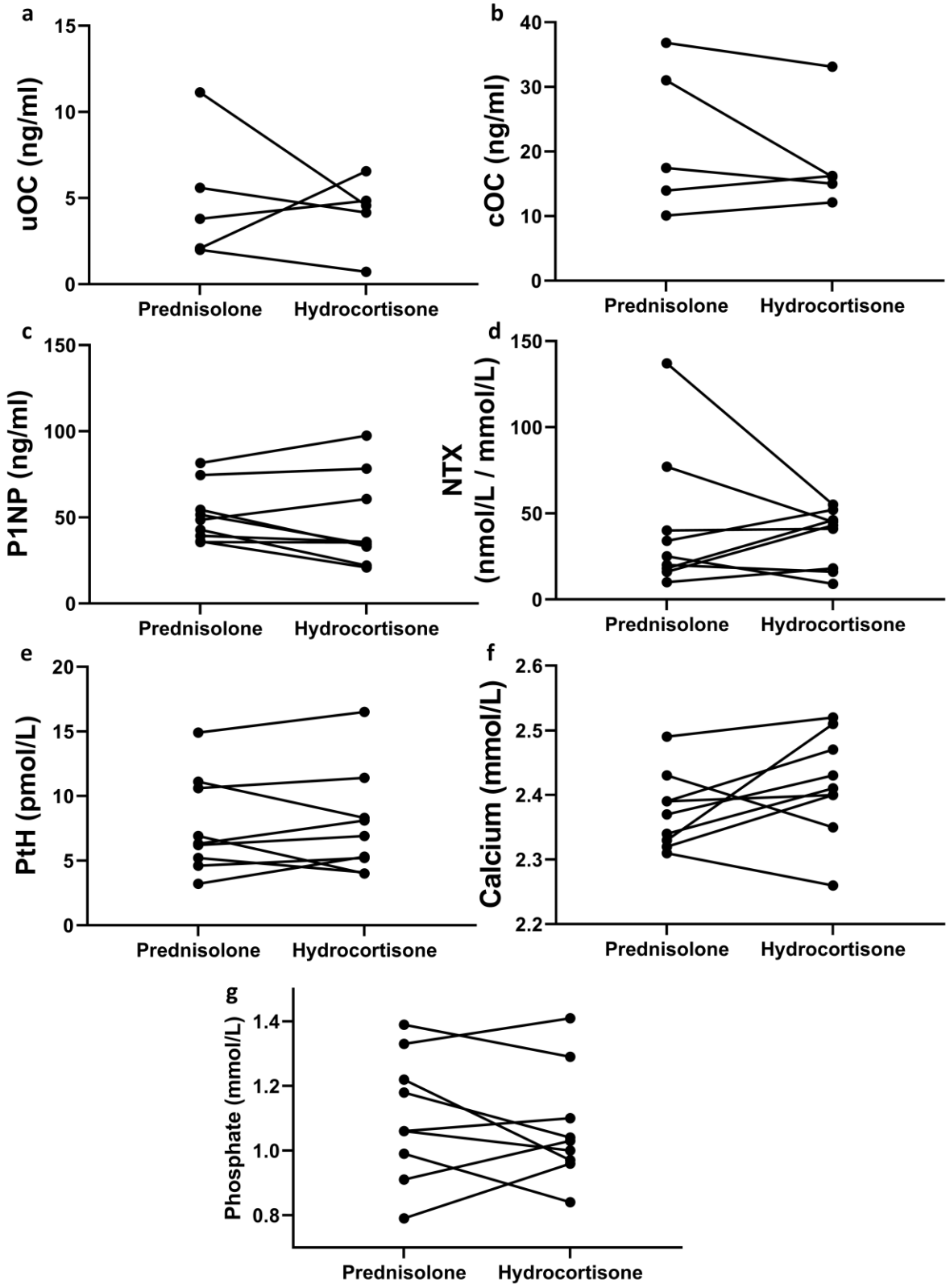

Figure 3.6- Changes in individual levels of undercarboxylated osteocalcin (uOC) (a), carboxylated osteocalcin (cOC) (b), procollagen type 1 N-terminal propeptide (P1NP) (c), N-terminal telopeptide (NTX) (d), parathyroid hormone (PTH) (e), calcium (f) and phosphate (g) between the same individuals on prednisolone and hydrocortisone. No significant differences between groups were seen.

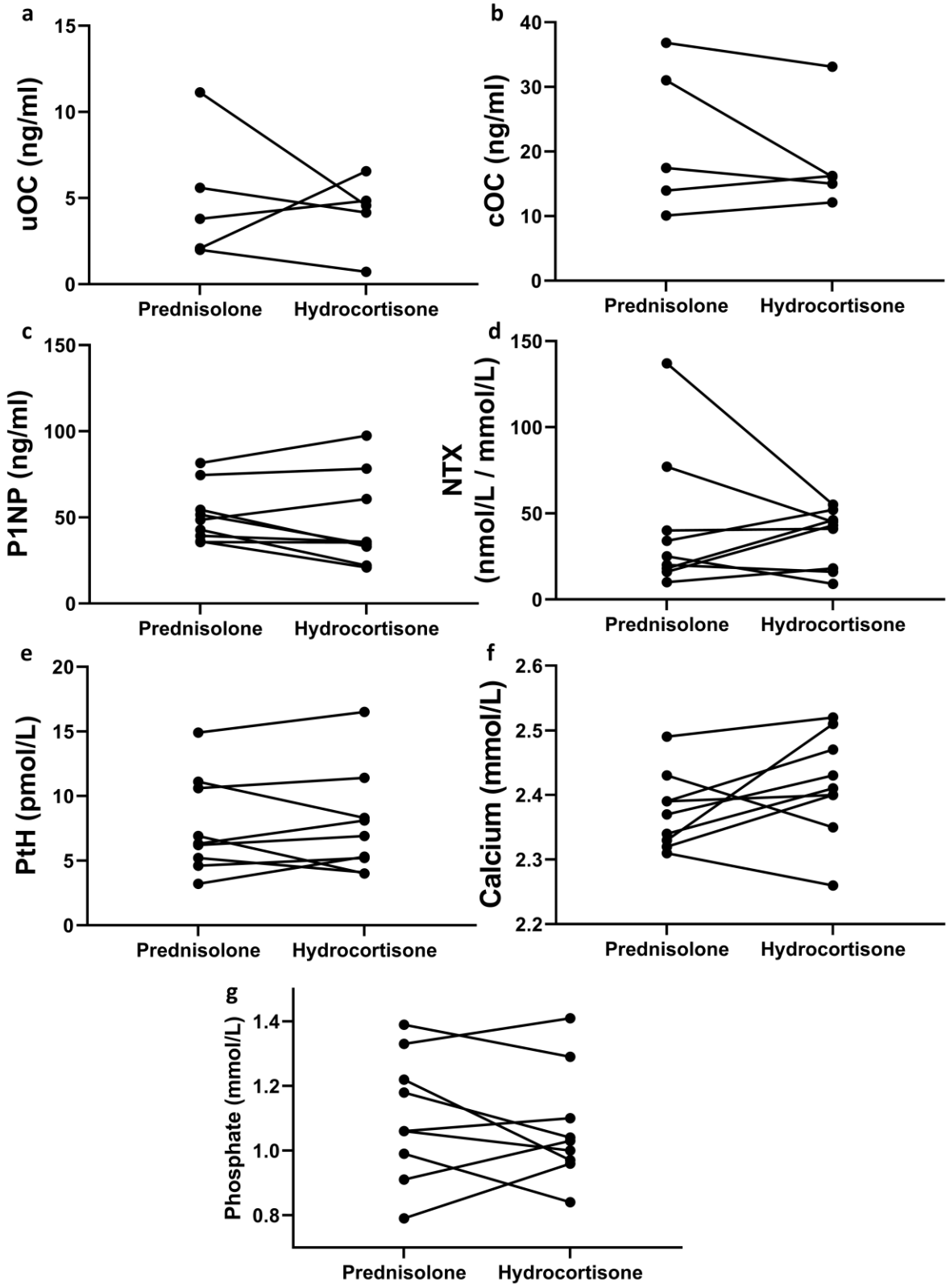

Figure 3.6- Changes in individual levels of undercarboxylated osteocalcin (uOC) (a), carboxylated osteocalcin (cOC) (b), procollagen type 1 N-terminal propeptide (P1NP) (c), N-terminal telopeptide (NTX) (d), parathyroid hormone (PTH) (e), calcium (f) and phosphate (g) between the same individuals on prednisolone and hydrocortisone. No significant differences between groups were seen.

3.4 Discussion

The data derived from the bone marker analysis principally shows that there is no significant difference in the primary outcome of uOC, cOC, OC ratios or total OC between the groups. This can be expected between the healthy volunteers and the AI patients, assuming that the AI patients are indeed sufficiently replaced, and not over-replaced. It is contrary to the expected findings for the high-dose glucocorticoids group.

A study of 7 healthy medical students given oral prednisolone 60mg daily for 5 days has previously shown that OC reduced within 24 hours of the first dose (184). OC levels remained suppressed for the entirety of the 5 days and returned to pre-treatment levels, 48 hours after the last dose was administered. Concordantly, a study of 9 asthmatic patients in receipt of acute infusions of betamethasone demonstrated a reduction in serum osteocalcin levels at 13 hours after the infusion (185). Of interest, 2 patients were already receiving 2.5 mg to 5 mg of prednisolone daily prior to their infusion. This is analogous to Groups B in the present study, and as in this study, their OC levels were comparable to the other 7 untreated patients, who are equivalent to Group A. In 4 individuals, the group characterised that OC levels remained suppressed 24 hours after cessation of the infusion and took up to 4 days to normalise. The data was unable to resolve the exact time of OC recovery as it was limited by the length of the blood sampling intervals.

On visual inspection of Figure 3.1, it is clear that the OC parameters reported appear to be lower in the high-dose group, but without reaching statistical significance. The high-dose group was the smallest of all the groups in the study and it is likely that the group was underpowered to show a difference. The data is further complicated by the study protocol which involved sampling blood 2 hours after the glucocorticoid infusion or tablet was administered. It is possible that this was too soon for OC to maximally respond in the IV infusion patients. The current literature shows changes at 12 hours, with 2 hours not explored. Further, the individuals receiving IV infusions were typically sampled

in the afternoon due to the time taken to prepare the methyl-prednisolone infusion in standard clinical care. This raises the prospect for the glucocorticoid induced suppression of OC to be mitigated by its diurnal rhythm which causes an afternoon peak (186). The large week-long gap between infusions may also have an impact. The transience of supressed OC is evidently 2 days, and the long interval between infusions likely permitted OC levels to normalise prior to patients returning for their next infusion.

Correlation analysis between glucocorticoid doses and OC parameters in Groups B and C, shows that whilst replacement regimens do not typically use large enough doses to overtly suppress OC, there are some more subtle dose dependent changes that are seen in OC levels. Greater doses of prednisolone appear to influence lower levels of uOC, which do not meet significance. As a percentage of the inactive form of OC, there is a significant negative correlation with OC-ratio. The consequence of this is not clear. More puzzling is that rising doses of hydrocortisone impact on cOC, and by extension total OC, showing another significant negative correlation.

Interpreted in the prism of our current understanding, these results imply that increasing doses of hydrocortisone are causing osteoblast suppression. This is not seen with prednisolone, but it is possible that high enough doses were not used in this study to cause the same effect. Prednisolone appears to have separate effect on reducing the active form of OC, uOC. Although steeped in uncertainty, this would indicate that prednisolone has a separate action of suppressing bone hormonal signalling. As elevated uOC is associated with augmented insulin secretion and insulin sensitivity, this finding provokes further correlation analysis of OC and insulin sensitivity later in this study (187).

Although unexpected, there is some precedence in these findings. While all glucocorticoids are assumed to only possess class effects, there is evidence of differential pharmacodynamic effects of

individual glucocorticoids. On an organ level, there is evidence of exclusion of some glucocorticoids such as cortisol and prednisolone from the central nervous system, in favour of corticosterone by the P-glycoprotein transporter (188). On a tissue level, in-vivo human studies have shown that each glucocorticoid has a preferential action on different tissue subsets, accounting for their different potencies (189). For instance, dexamethasone and prednisolone are better able to suppress lymphocytes than hydrocortisone. Yet in healthy individuals in the same experiment, prednisolone was better able to prevent eosinophil degranulation versus cortisol and dexamethasone. On a cellular level, hydrocortisone has been shown to bind and washout from the GR quicker than prednisolone or dexamethasone independently of concentration (82). Further differences have been noted in cellular transcriptomes after pulsing regimens of corticosterone versus constant stimulation.

The longer-term bone markers, P1NP and NTX did not show any significant differences between the groups. This indicates no difference between the healthy volunteers and the AI groups. Closer inspection of Figure 3.2 shows that the high-dose glucocorticoid group does trend towards a lower P1NP, without reaching significance and demonstrates scattered values for NTX. These results must be interpreted with care, as Group D was subject to a selection bias. The group had an overrepresentation of females with a median age of 55 years, the majority of whom are likely to have been post-menopausal with a trend towards elevated bone markers. The P1NP results may also have been impacted by the low sample number and the long interval between infusions. Individuals receiving prednisolone tablets in Group D, may not have received their tablets for a long enough period for P1NP to stabilise at a new baseline. In response to anti-resorptive therapy, P1NP has been shown to require 1 to 4 weeks to reacclimate to a new baseline and oral glucocorticoid course are seldom for greater than 1 week (190,191). The NTX data on the other hand, may have been affected by improper sampling. It was not possible to standardise the NTX sample collection in Group D, by virtue of their treatment regimen. As such, the urine samples were collected at various time, and the

results may reflect the natural diurnal rhythm of NTX, even in the context of glucocorticoid administration (192).

Calcium and phosphate levels were conserved between all groups, in keeping with homeostatic control. PTH was noted to be significantly elevated in the hydrocortisone group in the context of equivalent vitamin D levels between groups. The cause for this observation is speculative, especially as there is a known positive bias in the PTH assay used by NWLP in this study. Excess glucocorticoids have previously been characterised to reduce intestinal absorption and renal tubular reabsorption of calcium (193). This provokes an apparent secondary hyperparathyroidism picture, with associated compensatory elevation of 1,25α-vitamin D (194). More specifically, data from 6 males receiving at least prednisolone 7.5 mg daily for greater than 6 months has shown that whilst tonic secretion is significantly reduced, pulses of PTH become more frequent with greater amplitude (195).

This study was limited by an insufficient number of subjects, particularly in the high-dose glucocorticoid group. A cross-over study is better suited to detect subtle changes in bone marker, and ultimately quantitative CT or DEXA scans to assess BMD are the gold standard. The high-dose glucocorticoid group has also suffered as the majority of patients were receiving weekly infusions as opposed to daily glucocorticoid doses, meaning that they were “over-replaced” for a short period each week, as opposed to chronically over days. Sampling was also an issue in this group, as the sample collection could not be standardised as in the other groups. Unfortunately, this study did not contain enough patients in Groups B and C, who were crossing over between hydrocortisone and prednisolone.

In conclusion, this study shows no difference in bone turnover makers between the AI replacement groups. There are interesting observations of elevated PTH levels in the group taking hydrocortisone replacement compared to the healthy volunteers, and a demonstrable dose-dependent relationship

between hydrocortisone dose and total OC. Prednisolone appears to have an uncharacterised effect on bone signalling. The significance of these observations requires further investigation.

Chapter 4: The Effects of Different Glucocorticoid Regimens on Cardiovascular Risk

4.1 Introduction

Although the initial mortality associated with AI has been overcome with the advent of oral glucocorticoids, there still remains a mortality gap in which cardiovascular disease provides a significant contribution (27,29,196). Although a definitive link has yet to be proven, any previous use of glucocorticoids is associated with a 25% increased risk of cardiovascular events from case-control population studies (197). There is growing evidence that the excess mortality is associated with excess glucocorticoid exposure even from standard regimens that inherently provide too much glucocorticoid. Interrogation of UK GP practice data indicates an 86% increased risk of cardiovascular mortality for patients with PAI, and a 39% increase for patients with SAI (198). In past years, EU-AIR data has shown that deceased patients receive significantly higher doses of glucocorticoid than living patients, and other studies continue to show reduced mortality at increasingly lower doses of hydrocortisone (37,118,119).

Thirty-eight patients with Addison's disease were compared to 38 matched healthy controls (199). The patients with Addison's were receiving either cortisone acetate 37.5 mg or hydrocortisone 30 mg daily. Although lipid profiles, and glycaemic profiles including HOMA-IR and fasting glucose were not significantly different between the groups, there was a significantly greater proportion of patients in the Addison's group with marked central adiposity, hypercholesterolaemia and hypertriglyceridaemia.

Glucocorticoids have long been associated with blunting of the physiological nocturnal dipping of blood pressure and its negative effect on cardiovascular risk (200). In a study of 5 SAI patients, ambulatory blood pressure was noted to be significantly raised by treatment of hydrocortisone 15 - 25 mg once and twice daily (12 hour apart), and by prednisolone 3.75 – 5 mg once daily, compared to

the same individuals off-treatment (201). Of note, once daily hydrocortisone regimens provoked a significant nocturnal drop in mean and systolic blood pressure that was obliterated by twice-daily regimens and low-dose prednisolone. This suggests that preservation of the physiological nocturnal blood pressure dip may not be possible with oral glucocorticoid replacement without causing significant under-replacement.

Recent murine data has implicated the influence of CLOCK genes and the renal NaCl co-transporter in the mechanism of obliterating the nocturnal dip (202). NaCl co-transporter mRNA is modulated by PER1, and requires phosphorylation for activation. Peak activation of NaCl co-transporter correlates with peak levels of BMAL1 and PER2 levels which are in turn influenced by glucocorticoid exposure. Further, adrenalectomising mice reduced active NaCl co-transporter levels and nocturnal blood pressure dipping, without changing the expression of the CLOCK genes. The research suggests that thiazide diuretics as a good treatment option to restore nocturnal blood pressure variation in patients prescribed glucocorticoid replacement.

The association of glucocorticoid replacement and visceral adiposity is less clear (203). Parallels are routinely drawn with Cushing's syndrome, in which central and visceral adiposity is a defining characteristic with a strong association with an adverse cardiovascular risk profile through its effects on dyslipidaemia and glycaemic handling (204-206). A cross-sectional case-control study completed a cardiovascular metabolic risk profile and CT imaging to map body composition and distribution of adiposity in 76 AI patients and 76 health controls (203). Urinary cortisol collections verified significantly higher glucocorticoid exposure in the patient group but did not demonstrate any differences in blood pressure, or body composition. Serum triglyceride levels were higher in the AI group compared to the volunteers, and HDL was lower after exclusion of individuals on lipid modifying medication. Lastly, a number of pro-inflammatory markers including interleukin-6 were elevated in the patient group compared to the controls. This study shows that the apparent difference in

cardiovascular risk is not easy to detect using the overt measures used in usual clinical practice. An approach to characterise risk in a short-term study must therefore use a number of more subtle markers to show variance in risk between different treatments in AI and between patients and controls.

A study of 17 patients with AI on a median dose of 33.5 mg cortisone acetate compared to 17 matched healthy controls demonstrated echocardiographic differences between the groups (207). In particular, the AI group had significantly smaller left ventricular end diastolic diameter, left atrial diameter and elevated ejection fraction. The AI group biochemically showed a significantly higher total cholesterol, and had lower nocturnal blood pressure, with other parameters being comparable. After 10 individuals switched to an equivalent dose of Plenadren, a significant reduction in LDL and total cholesterol was observed. Total cholesterol levels were in fact now no longer different from levels in healthy controls. Of note, nocturnal diastolic blood pressure increased to healthy control levels. There were no changes in the echocardiographic findings. The study did not involve randomisation and is therefore confounded by selection bias. Previous data has shown that conversion to an equivalent MR-HC dose leads to an approximate 20% reduction in total glucocorticoid exposure (101). This highlights the complicated and incomplete understanding of the relationship between glucocorticoids and cardiovascular risk. Whilst it is clear, that 20% reduction in exposure can provoke reductions in total cholesterol and LDL, it is unclear why it should have a detrimental effect on nocturnal blood pressure. It is possible that the pattern of replacement with MR-HC leads to greater levels of glucocorticoids in the system later in the day when the body is most sensitive, but this has not been clearly demonstrated. Further this study demonstrates the difficultly in characterising cardiovascular risk at a single timepoints between AI patients and controls in the absence of imaging.

For a single timepoint observational study, in the absence of long-term prospective data, the options for cardiovascular risk markers are limited. Blood pressure and heart rate are obvious selections with

the long term evidence of hypertension being associated with cardiovascular disease and mortality (208-210). There is high quality evidence showing pharmacological reduction of systolic and diastolic blood pressures have a substantial effect on reducing myocardial infarctions, strokes and arterial disease (211-213). Similar large population studies have demonstrated the well characterised relationship with higher resting heart rates and ischaemic heart disease and death (214-216). Every 10 beats per minute (bpm) rise above 60 bpm is associated with an approximate 20% increased chance of cardiovascular death (217).

The literature concerning the link between weight, BMI and cardiovascular disease is controversial, with the reports of an "obesity paradox" (218). This has been sparked by data in the early 2000's including a meta-analysis of nearly 3 million people, which reported an 18% increase in all-cause mortality in obese individuals with a BMI of >30 kg/m^2, but no increase in mortality for overweight individuals with BMIs of 25 - <30 kg/m^2 (219). Further, the same study also showed no increase in hazard ratio for individuals with grade 1 obesity with a BMI of >30 - <35 kg/m^2. There are a number of important confounding factors. The study did not consider the effect of ethnicity on the BMI association with adverse outcomes. It further, does not distinguish that all-cause mortality includes other causes of death in addition to cardiovascular causes. Despite this, there is also a body of evidence showing the opposite. This includes a population-based study including 3.2 million person-years of data that showed a 21% and 32% increase in risk of cardiovascular incidents for overweight and grade-1 obese people (220). The medical consensus with this body of evidence remains that raised BMI is associated with an adverse cardiovascular profile, especially in light of its association with metabolic syndrome and development of diabetes (221). Alongside waist-hip ratios, BMI is therefore still considered as an integral parameter to measure.

To further quantify cardiovascular risk, haematological markers including hs-Trop, hs-CRP and BNP have shown promise (222-225). Hs-Trop has proven valuable beyond its use in early diagnosis of acute

coronary syndrome, to indicate cardiovascular risk whilst in the reference range (226,227). In approximately 75,000 participants from prospective studies, hs-Trop has correlated well with rates of cardiovascular death, disease, and all-cause mortality (228). A further prospective study followed patients up for up to 7 years after quantifying hs-Trop levels, with MRI imaging (229). Higher levels of hs-Trop were associated with left ventricular hypertrophy, systolic dysfunction and chronic kidney disease (CKD). Mortality was up to 15-times higher in the patients with the highest levels versus the lowest. The study also found that hs-CRP and N-terminal pro-BNP (NT-proBNP) were independently associated with mortality. Importantly, hs-Trop has also been shown to decrease when individuals are treated with Pravastatin, indicating that it does correlate with decreasing cardiovascular risk within individuals (230).

Hs-CRP and BNP are not routinely used as primary endpoints in clinical studies, out of favour for stronger measures of risk. Early data from the Framingham Heart Study demonstrated up to a 60% increase of risk for cardiovascular disease in individual with a CRP >3 mg/L (231). A meta-analysis of 54 prospective studies and 160,000 people showed that logged concentrations of hs-CRP are linearly corelated with other cardiovascular risk factors, markers of inflammation and cardiovascular death (232). Building on this, the JUPITER study utilised hs-CRP and LDL levels as inclusion criteria to assess the efficacy of rosuvastatin (233). The study was terminated early given the 44% reduction in adverse cardiovascular outcomes seen in the rosuvastatin group, but it served to show that statins caused a concurrent reduction of 37% in hs-CRP highlighting its use as a dynamic marker of cardiovascular risk (234).

NT-proBNP and BNP have been considered interchangeably in this manuscript but are in fact, distinct. Both peptides are formed from the cleavage of the precursor molecule, proBNP in equimolar concentrations. BNP is the biologically active molecule, whilst NT-proBNP is the inactive N-terminal fragment. NT-proBNP is a more robust marker, with greater stability and evidence for its use

compared to BNP in this context (224). Although NT-proBNP is more commonly used in the relevant studies performed to date, it strongly correlates with BNP. NT-proBNP is preferred as a cardiac marker, but this study was limited to using BNP as this is the analyte measured at ICHNT. Nevertheless, a metanalysis of over 95,000 apparently healthy individuals from 40 prospective studies has demonstrated a non-linear positive correlation with heart disease, heart failure and stroke (235). Within the reference range, the top tertile of NT-proBNP showed a 76% increase in risk for heart disease and stroke versus the lowest tertile.

It is unlikely that the OMNI-AID study will show specific differences in the cardiovascular risk markers secondary endpoint, between Groups A-C. There is no evidence that these markers are sensitive enough to pick up the subtle differences between AI regimens and healthy volunteers as evidenced by the lack of studies reporting significant findings in these biomarkers for these populations. The absence of a difference remains an important finding to show that there is no overt detriment associated with either treatment for AI. All cardiac markers will be directly compared between all 4 groups.

4.2 Hypotheses and aims

4.2.1 Hypotheses

1- There will be no significant difference in the non-biochemical or biochemical markers of cardiovascular risk between Groups A-C.
2- Patients receiving high doses of glucocorticoids (Group D) will show elevations in selected anthropometric and biochemical biomarkers.

4.2.2 Aims

To compare anthropometric and biochemical markers of cardiovascular risk between patients prescribed hydrocortisone and prednisolone replacement regimens in AI, as well as with healthy volunteers and patient receiving high doses of glucocorticoids.

4.3 Results

4.3.1 Baseline demographic data

Table 4.1 shows the number of individuals being managed for hypertension and hyperlipidaemia in each group.

	Group A Healthy Volunteers n=20	**Group B Prednisolone n=20**	**Group C Hydrocortisone n=20**	**Group D High Dose n=9**	**χ2 Test**
Number receiving anti-hypertensives	0	4	6	1	0.067
Anti-hypertensives used	N/A	Perindopril x1 Amlodipine x1 Ramipril x1 Losartan x1	Perindopril x1 Amlodipine x4 Ramipril x1 Losartan x1 Verapamil x1 Propranolol x1 Lisinopril x1	Doxazosin x1	
Number of anti-hypertensives per participant	N/A	2 individuals on 1 agent 1 individual on 2 agents	3 individual on 1 agents 2 individual on 2 agents 1 individual on 3 agents	1 individual on 1 agent	
Number receiving lipid-lowering therapy	1	3	8	1	0.031
Lipid-lowering therapy used	Atorvastatin x1	Atorvastatin x3	Atorvastatin x4 Simvastatin x4	Simvastatin x1	

Table 4.1- Baseline pharmacological treatment for in participants in the study.

4.3.2 Anthropometric markers

Mean (±SD) SBP was 121 (±17), 125 (±19), 132 (±17) and 128 (±12) mmHg for Groups A-D respectively. Although patients receiving hydrocortisone had higher blood pressures than the healthy volunteers, this did not reach significance. DBP, MAP and HR were also comparable between groups (Figure 4.1). The same analysis of these parameters was completed after excluding all individuals taking antihypertensive therapy. This yielded the same results as the initial analysis.

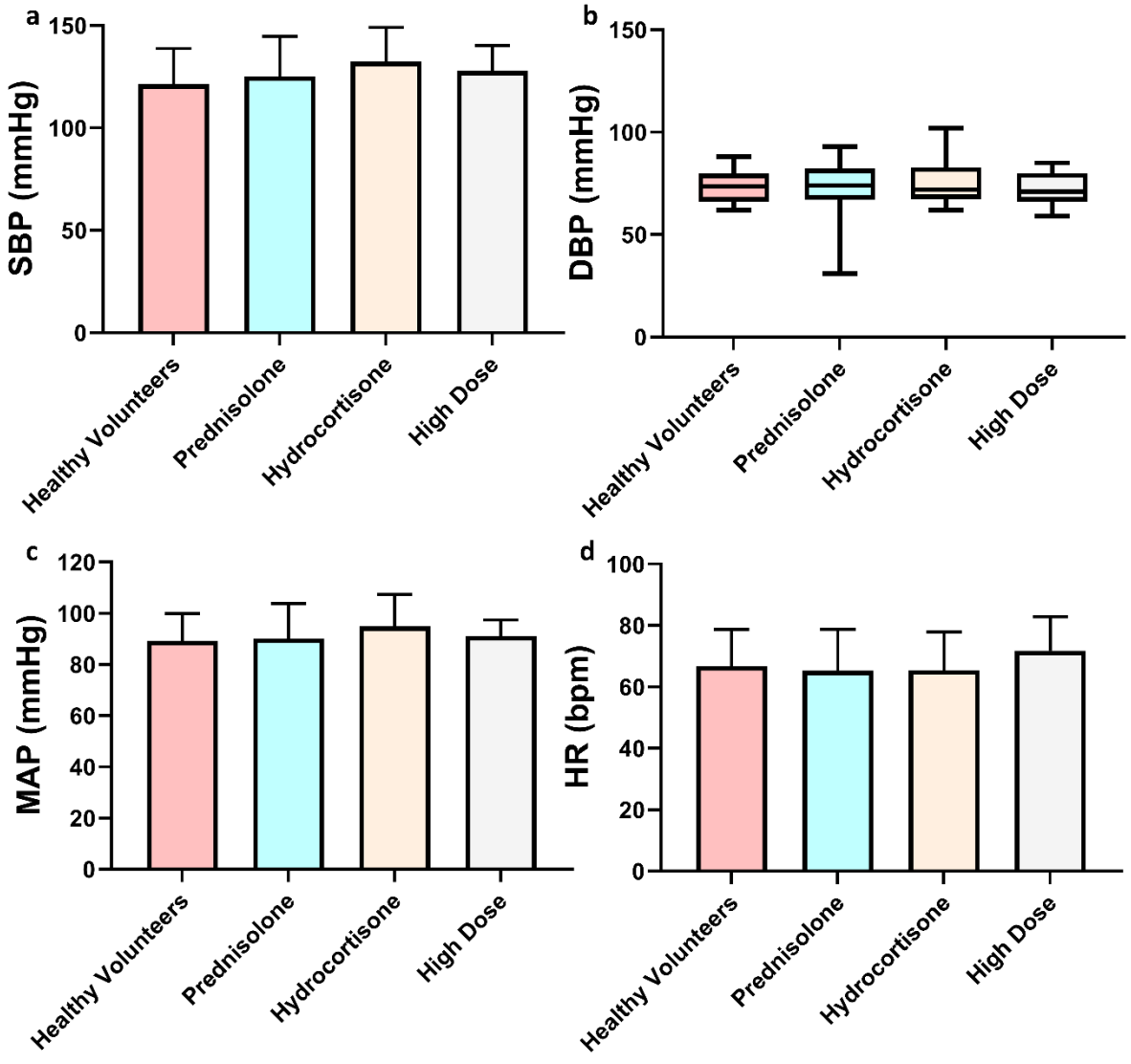

Figure 4.1- Differences between groups in systolic blood pressure (SBP) (a), diastolic blood pressure (DBP) (b), mean arterial pressure (MAP) (c) and heart rate (HR) (d). Data is given as median, quartiles and range in (b). Data is given as mean + SD in (a), (c) and (d). Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=20; high dose n=9. No significant differences between groups were seen.

Median (IQR) weight was 71.4 (13.2), 67.7 (11.9), 75.7 (24.4), 63.5 (10.9) kg in Groups A-D respectively. Kruskal-Wallis analysis showed significance (P=0.04), however post-hoc multiple comparisons analysis did not show an interaction between groups (Figure 4.2). Mean WHR was significantly higher in the AI participants taking prednisolone and hydrocortisone versus the healthy volunteers (ANOVA P=0.009). Mean (±SD) WHR was 0.83 (±0.07), 0.90 (±0.09), 0.90 (±0.07) and 0.84 (±0.09) in Groups A-D respectively. Median fat mass was significantly elevated in the hydrocortisone group compared to the healthy volunteer at 22.0 (9.5) kg versus 15.3 (5.7) kg (P=0.025). There was no difference between groups in BMI, % fat mass or % lean mass (Table 4.2).

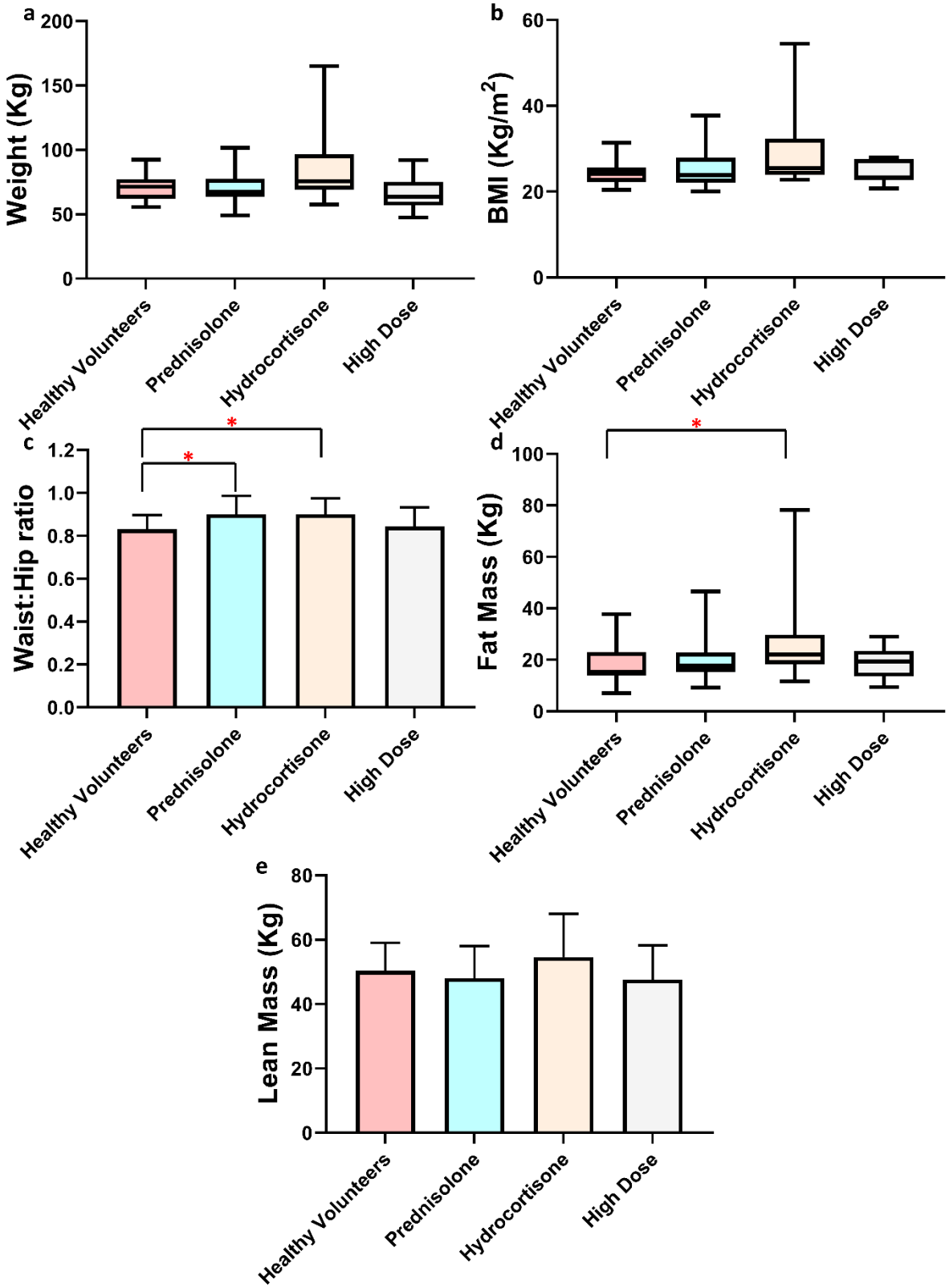

Figure 4.2- Differences between groups in weight (a), body mass index (BMI) (b), waist-hip ratio (WHR) (c), fat mass (d) and lean mass (e). Data is given as median, quartiles and range in (a), (b) and (d). Data is given as mean + SD in (c) and (e). Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=20; high dose n=9.* denotes significance P<0.03

Parameters	Group A Healthy Volunteers	Group B Prednisolone	Group C Hydrocortisone	Group D High Dose	Significance
SBP (mmHg)	121 (±17)	125 (±19)	132 (±17)	128 (±12)	P=0.26
DBP (mmHg)	74 (13)	74 (14)	72 (15)	71 (9)	P=0.91
MAP (mmHg)	89 (±11)	90 (±14)	95 (±12)	91 (±6)	P=0.44
HR (bpm)	67 (±12)	65 (±13)	63 (±22)	72 (±11)	P=0.59
Weight (kg)	71.4 (13.2)	67.7 (11.9)	75.7 (24.0)	63.5 (10.9)	P=0.04*
BMI (kg/m^2)	24.2 (2.8)	23.9 (5.4)	25.5 (6.8)	23.3 (4.8)	P=0.11
WHR	0.83 (±0.07)	0.90 (±0.09)	0.90 (±0.07)	0.84 (±0.09)	P=0.009*
Fat Mass (kg)	15.3 (5.7)	17.7 (6.6)	22.0 (9.5)	19.4 (8.9)	P=0.04*
Fat Mass (%)	25.2 (±8.7)	28.1 (±7.9)	30.7 (±8.6)	27.8 (±6.1)	P=0.22
Lean Mass (kg)	50.4 (±8.7)	48.1 (±10.0)	54.6 (±13.4)	47.6 (±10.6)	P=0.22
Lean Mass (%)	71.0 (±8.3)	68.3 (±7.5)	66.0 (±8.2)	65.6 (±11.1)	P=0.23

Table 4.2- Anthropometric cardiovascular risk data for Groups A-D. Data is reported as mean (±SD) for parametric data and median (IQR) for non-parametric data. Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=20; high dose n=9. * denotes significant P-values.

4.3.3 Biochemical markers

There was no significant difference between groups in hs-Trop or BNP. For the purposes of analysis, values of hs-Trop reported as <2 ng/L were taken as 0 ng/L. Of note, median (IQR) hs-CRP was significantly elevated in the hydrocortisone cohort at 4.4 (1.4) mg/L compared to 3.6 (2.4) mg/L in the healthy volunteers (P<0.01) (Figure 4.3). Potassium concentration was noted to be reduced in both the hydrocortisone and high dose groups, at 4 (0.4) mmol/L (P=0.03) and 4 (0.4) mmol/L (P=0.02) compared to 4.2 (0.2) mmol/L in the healthy controls.

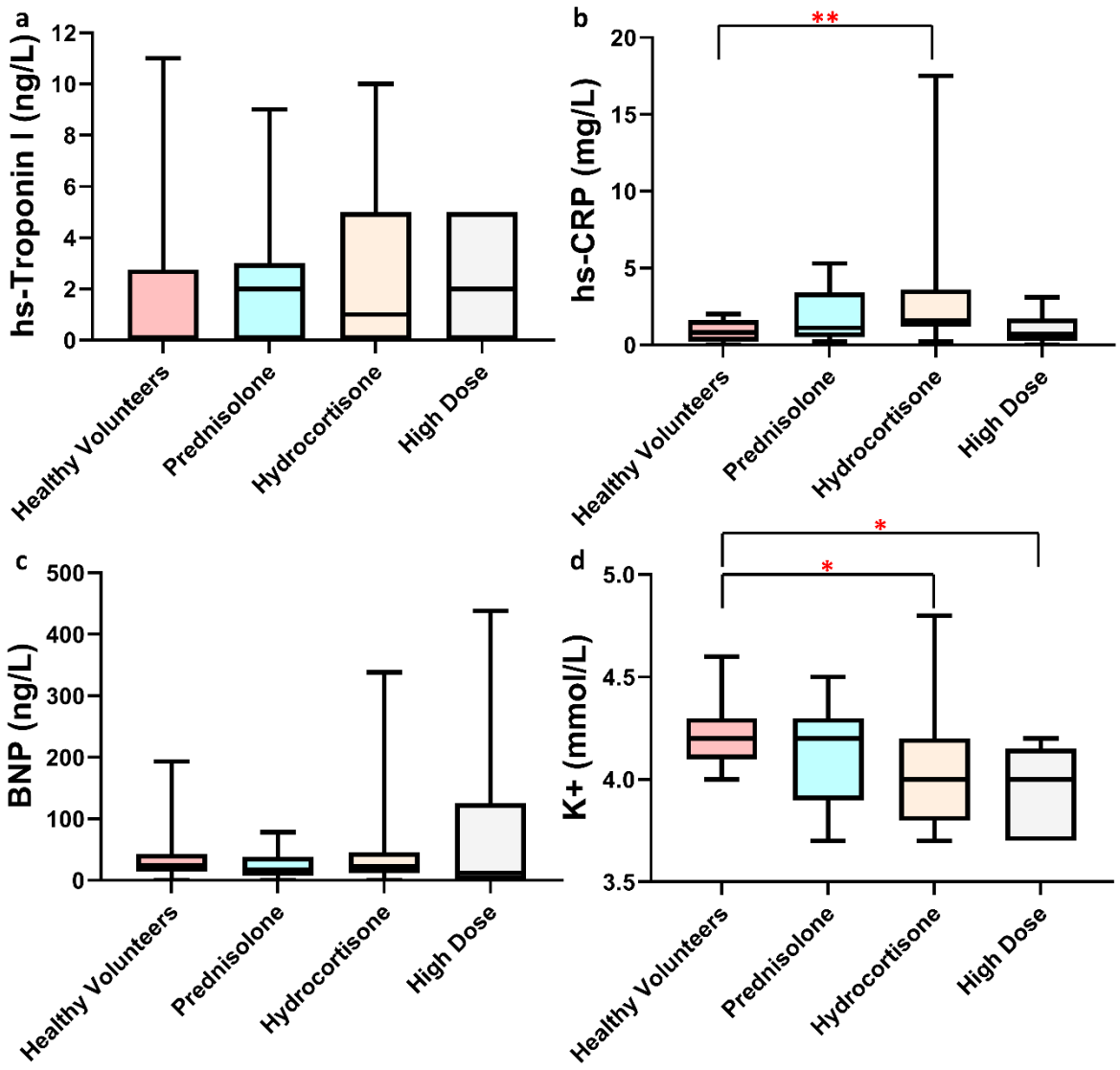

Figure 4.3- Differences between groups in high sensitivity (hs)-Troponin I (a), hs-CRP (b), brain natriuretic peptide (BNP) (c) and potassium (K+) (d). Data is given as median, quartiles and range. Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=19; high dose n=9. *denotes P<0.03 **denotes P<0.01.

Lipid profile data was analysed before and after exclusion of individuals taking lipid lowering medication. The outcomes of both analyses were the same. The data presented herein includes all participants including those receiving statins. The only significant finding was that the median (IQR) triglyceride levels in patients prescribed hydrocortisone were significantly elevated compared to the

healthy controls at 1.4 (1.0) mmol/L compared to 0.86 (0.4) mmol/L (Figure 4.4). No differences were detected in the other lipid differentials (Table 4.3).

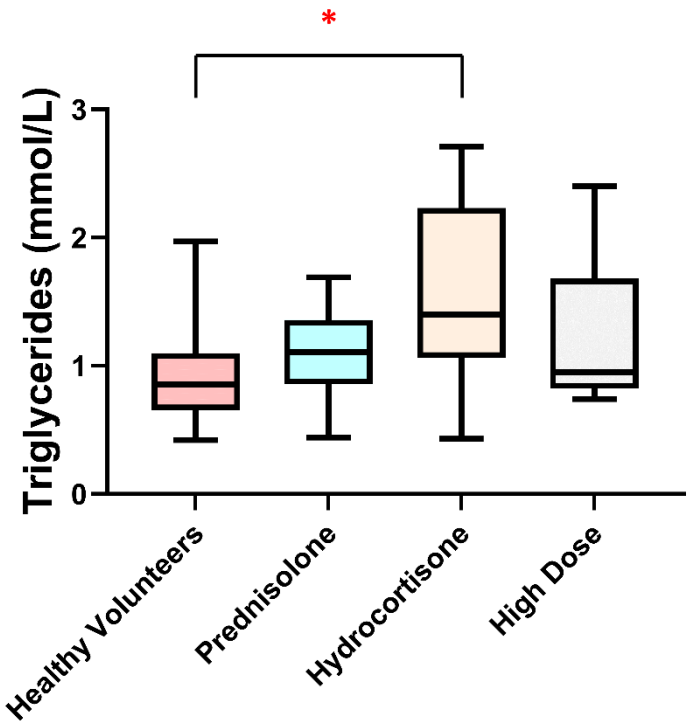

Figure 4.4- Differences between groups in triglyceride levels. Data is given as median, quartiles and range. Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=19; high dose n=9. *denotes P=0.005

Measurand	Group A Healthy Volunteers	Group B Prednisolone	Group C Hydrocortisone	Group D High Dose	Significance
hs-Trop (ng/L)	0 (2.3)	2 (3.0)	1 (5.0)	2 (5.0)	P=0.96
hs-CRP (mg/L)	0.8 (1.1)	1.1 (2.5)	1.6 (2.1)	0.7 (0.9)	P=0.008*
BNP (ng/L)	24.5 (26.8)	17.0 (25.5)	22.5 (30.8)	12.0 (27.0)	P=0.70
Potassium (mmol/L)	4.2 (0.2)	4.2 (0.4)	4.0 (0.4)	4.0 (0.4)	P=0.005*
Total cholesterol (mmol/L)	5.3 (±1.3)	5.2 (±1.1)	5.1 (±1.3)	5.9 (±1.6)	P=0.46
Triglycerides (mmol/L)	0.86 (0.40)	1.11(0.49)	1.40 (0.99)	0.95 (0.67)	P=0.01*
HDL (mmol/L)	1.56 (±0.38)	1.58 (±0.41)	1.43 (±0.32)	1.70 (0.34)	P=0.31
LDL (mmol/L)	3.30 (±1.20)	3.26 (±0.79)	2.96 (±1.15)	3.61 (±1.23)	P=0.50
Total Cholesterol: HDL Ratio	3.5 (±1.0)	3.6 (±1.0)	3.7 (±1.1)	3.5 (±0.7)	P=0.95
Non-HDL (mmol/L)	3.73 (±1.23)	3.74 (±0.78)	3.65 (±1.26)	4.12 (±1.39)	P=0.70

Table 4.3- Biochemical cardiovascular risk data for Groups A-D. Data is reported as mean (±SD) for parametric data and median (IQR) for non-parametric data. Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=19; high dose n=9. * denotes significant P-values. hs-Trop- high sensitivity troponin I; hs-CRP- high sensitivity CRP; BNP- brain natriuretic peptide; HDL- high density lipoprotein; LDL- low density lipoprotein.

4.3.4 Data from crossover analysis

Nine individuals in the study crossed over from either prednisolone to hydrocortisone or vice versa for a minimum of 4 months. All cardiac markers in this study were compared in a paired analysis, in the 9 patients. With the exception of potassium, none of the biomarkers showed a significant

difference between the two treatments (Figure 4.5). The mean (±SD) potassium during prednisolone treatment was 4.2 (±0.3) mmol/L compared to 4.0 (±0.3), during hydrocortisone (P=0.04).

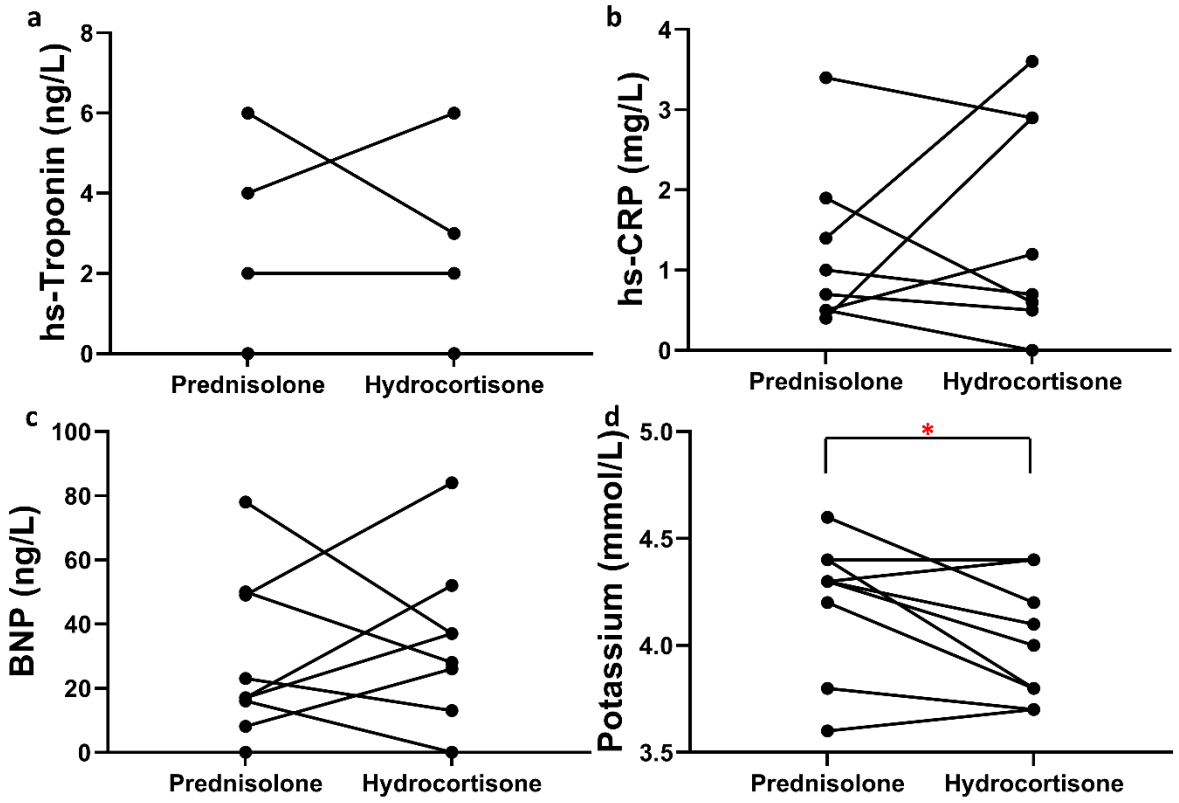

Figure 4.5- Changes in individual levels of high sensitivity (hs)-troponin I (a), hs-CRP (b), brain natriuretic peptide (BNP) (c), and Potassium (d) between the same individuals on prednisolone and hydrocortisone. Six individuals had no change in hs-troponin levels, from a baseline of <2 ng/L; n=9. *denotes P=0.04.

4.4 Discussion

Analysis of the cardiovascular parameters measured in this study suggests that hydrocortisone therapy is associated with a worsened cardiovascular profile compared to healthy volunteers. There are further signals of greater adverse metabolic risk in both AI groups versus the healthy volunteers, but no evidence of overt differences between prednisolone and hydrocortisone replacement.

A broad look at the number of individuals receiving antihypertensives shows that AI groups contain more individuals diagnosed with hypertension. The data is suggestive and trends towards significance without achieving it. The number prescribed lipid lowering therapy, were significantly higher in the hydrocortisone group.

In the context that there was no difference in blood pressures between any of the groups, with and without hypertensive patients included, it is possible that the greater number of diagnoses in the AI groups is because of closer monitoring. AI patients will have at least yearly follow ups with routine blood work and measurement of anthropometric parameters (49). Healthy volunteers are less like to be diagnosed given the reduced surveillance.

The significantly elevated fat mass in the hydrocortisone group, and WHR in both AI groups certainly indicates that there is an increased metabolic burden in AI. Adding to this, individuals receiving hydrocortisone demonstrated higher hs-CRP, triglycerides and lower potassium levels compared to the healthy volunteers. Potassium levels are significantly lower in patients taking hydrocortisone compared to when they are taking prednisolone as shown in the paired analysis. More individuals in the hydrocortisone group were also prescribed ACE inhibitors and ARBs, which tend to elevate potassium levels and should have caused a negative bias. Yet the differences in potassium levels remained detectable. These findings must be accepted cautiously, as hs-Trop and BNP did not show concordant changes. Hs-Trop analysis was however complicated by the large number of participants who had levels that were less than the LLOQ of the assay, implying that the assay was not sensitive enough. Taken together, these findings hint towards hydrocortisone therapy causing a worsened cardiovascular profile compared to healthy volunteers.

The increased WHR in the AI groups is in keeping with the current literature. A study of 2424 patients with hypopituitarism has previously demonstrated an increased waist circumference in

hydrocortisone treated patients and increased WHR in prednisolone patients, when compared to healthy volunteers (75). It is noteworthy that the mean prednisolone dose used in the study was 6.7 mg compared to 3.3 mg in the present study.

The increased fat mass in the hydrocortisone group is suggestive of glucocorticoid over-replacement. Eleven patients who had their hydrocortisone doses halved from 20-30 mg/day to 10-15 mg/day demonstrated an average loss of 7.1 kg of fat mass in under 1 year (236). The study demonstrates that changes in fat mass are sensitive enough to pick up, and suggests that this study was adequately powered to pick up the significant difference between Groups A and C.

The triglyceride data provides further suggestive evidence of over-replacement in the hydrocortisone group, more so than in the prednisolone group. A double-blind study of 32 healthy volunteers given either placebo, prednisolone 7.5 mg or prednisolone 30 mg daily for 2 weeks showed that fasting triglycerides were significantly elevated by the 30 mg dose but not 7.5 mg (237). Although it is not possible to comment on the effect that 7.5 mg of prednisolone would have had in the long-term, it is clear that the class effect of excess glucocorticoids is to raise triglycerides in the subacute setting at the least.

Although potassium was not initially included in the study protocol as a cardiovascular outcome, this was an oversight in the study design and became apparent in early interim analyses comparing 10 hydrocortisone and prednisolone patients against 20 healthy volunteers. By this point, potassium was already significantly lower in the hydrocortisone group. Potassium levels are traditionally thought to be regulated via aldosterone signalling that stimulates renal secretion through activating the mineralocorticoid receptor (238). Renal 11-HSD2 prevents glucocorticoid-mediated mineralocorticoid receptor activation by converting cortisol and prednisolone into cortisone and prednisone respectively (55). High doses of glucocorticoids can overwhelm the enzymatic activity of 11-HSD2 and activate the

mineralocorticoid receptor. Glucocorticoid levels in replacement regimens are not sufficient to completely replace mineralocorticoid requirements, as evidenced by the need to supplement aldosterone deficient Addison's patients, with fludrocortisone. However, studies have previously demonstrated that physiological doses of hydrocortisone can cause kaliuresis, in both basal and potassium chloride stimulated states, in hypopituitary and Addisonian patients (239,240). Further, glucocorticoids such as dexamethasone and betamethasone have been shown to cause hypokalaemic periodic paralysis, noting that they have very little activity at the mineralocorticoid receptor and suggesting that glucocorticoids have a mineralocorticoid-independent effect on kaliuresis (241). This direct renal mechanism of action exerted by glucocorticoids is not yet fully understood.

Even in the normokalaemic range, there is growing evidence of increased cardiovascular mortality with seemingly mild fluctuations away from 4.5 mmol/L. A systematic review of 123 studies showed a "U"-shaped distribution of cardiovascular mortality as potassium deviated from 4.5 to 5 mmol/L in heart failure patients, dipping to a hazard ratio of 1.53 between 3.5 and 4.0 mmol/L (242). A study of 90-day mortality in approximately 45,000 individuals with hypertension showed that even potassium levels between 3.8 to 4.0 mmol/L was associated with an increased all-cause mortality of 21% compared to levels 4.5 to 5.0 mmol/L. This increased to 70% at 3.5 to 3.7 mmol/L (243). It is important to note that findings of the above studies show associations between potassium levels and mortality in specific disease populations as opposed to healthy individuals. It is unclear how this relates to AI populations or whether this is a genuine marker of increased cardiovascular risk in the hydrocortisone cohort.

Studies involving MR-HC have demonstrated that cardiovascular markers should be sensitive enough to detect 20% reductions in glucocorticoid exposure. Conversion from conventional glucocorticoids to MR-HC has shown significant reductions in WHR, LDL, SBP and weight (104,108,126). Given that only WHR showed significance, there are 3 possibilities. Firstly, it is possible that WHR is the most sensitive

marker and the first to pick up significance differences between replacement regimens. Secondly, there may not be a difference of up to 20% glucocorticoid exposure between Groups A to C. Thirdly, and most likely, the study was underpowered to detect significant differences in the other cardiovascular parameters. In keeping with the third possibility, although the multiple comparisons analysis of weight indicated a significant interaction, this was not the case in the post-hoc analysis. The most likely cause for this is the hydrocortisone group having a greater median weight compared to the healthy volunteers, that did not quite reach significance due to the study being under-powered.

Further limitations that impact interpretation of these results include the limited time individuals had after crossing over between hydrocortisone and prednisolone or vice versa. The crossover analysis has likely suffered as most individuals had only swapped treatments for 4 months. It is clear from the literature, that some of the markers such as BMI, WHR and LDL may require 6-12 months to adopt a new measurable baseline.

Another limitation is that this study does not consider the *a priori* risk of cardiovascular disease in any of the participants. The interpretation of the results hinges on the central assumption that increased glucocorticoid dose and therefore exposure, will increase cardiovascular risk and markers of risk. It has been well characterised that patients with PAI in South Africa have a worse cardiometabolic profile compared to their unaffected healthy counterparts (244). In particular, they have significantly elevated triglycerides, hs-CRP, LDL and have reduced HDL. When these individuals were however compared with matched AI patients in Sweden, the same significantly adverse pattern of cardiovascular markers emerged (245). This was despite the Swedish participants receiving an average of 33.0 mg of hydrocortisone compared to 24.3 mg by their Caucasian South African counterparts. This highlights the risk of recruiting a homogenous population with a shared genetic risk profile. This is unlikely to have occurred in this study given the heterogenous nature of the London population, but the potential for this effect was not accounted for.

The most important limitation is the design of the study itself. This study has drawn conclusions based on surrogate markers of cardiovascular risk only. Evidence is required to substantiate the impact on clinically significant outcomes such as the number of individuals diagnosed with ischaemic heart disease, or cardiovascular death. The nature of this study, as a principally single timepoint observational study, makes it impossible to use these clinical outcome measures. Other measures such as nocturnal blood pressure, could not be measured in the study but would be useful in future trials. Given that this study has so far shown that there is some evidence of differences between prednisolone treatment, hydrocortisone treatment and healthy volunteers, without any immediate concerns of detrimental effects, I believe there is justification for a longer-term study measuring major adverse cardiovascular events (MACE) outcomes. So far, there are only registry-based retrospective studies looking at epidemiological outcomes. There is a distinct absence of prospective large population or community studies that follow AI patients and healthy individuals over a period of 10 or more years. Such a study is now desperately needed.

Chapter 5: The Effects of Different Glucocorticoid Regimens on Glycaemia

5.1 Introduction

A Swedish observational cohort study compared 226 patients with Addison's disease and diabetes with 1129 individuals with diabetes alone (246). Not only did the patients with Addison's and diabetes suffer a greater frequency of diabetic complications, but they were also experienced 3.89-fold greater risk of all-cause mortality.

These finding are in keeping with the catabolic actions of glucocorticoids, particularly on proteolysis which in turn drives gluconeogenesis, in addition to glucocorticoid induced increased hepatic glucose output (247). Further direct action of glucocorticoids in suppressing insulin secretion in islet β-cells may further compound the process (144,248). A hyperinsulinemic clamp study of healthy volunteers showed that 7.5 mg of daily prednisolone for 2 weeks is enough to cause significant insulin resistance (237). This was characterised by decreased insulin suppression of glucose production, lipolysis and increased tendency for proteolysis. This shows that there are detectable changes in metabolism after a relatively short period of glucocorticoid treatment in the absence of diabetes, albeit with a relatively large dose of prednisolone.

Other clamp studies have not been so successful in demonstrating significant changes (249,250). Further, there are a paucity of studies that sufficient power in other markers such as HOMA measures and insulin levels to demonstrate differences between regimens. This is despite good agreement in the literature between clamp studies and these indices. A study of 17 patients with SAI who received a 7-day increase in their hydrocortisone replacement regimen from 20 mg per day to 30 mg per day, did not demonstrate a difference in fasting or 2 hour glucose, insulin or insulin secretion and insulin resistance indices (124). This is in keeping with other similar studies (236,251).

A more robust measure of change is HbA1c. HbA1c involves quantification of glycated haemoglobin and is therefore a good measure of glycaemic levels over the preceding 4 months. A study of 10 PAI and 9 SAI patients who were prospectively tracked in 3-monthly intervals over a year after changing treatment to MR-HC showed significant reduction in HbA1c, after 1 year (252). Unfortunately, data showing the minimum time-period needed for the change in HbA1c to become detectable and the magnitude of change were not published. An earlier study into MR-HC, did show that significant changes of -0.1% HbA1C were detectable at 4 months, in 64 individuals enrolled in a cross-over study comparing MRHC and conventional glucocorticoids (101). These papers serve to show that HbA1c can be a useful marker to examine glycaemic changes between treatments.

In addition to fasting glucose levels, as a short-term measure of changes in glycaemic handling, fructosamine was also measured in this study. Fructosamine involves the quantification of proteins, including albumin, that have been glycated due to the presence of serum fructose or glucose (253). Fructosamine is a measure of glucose control over the preceding 2 to 3 weeks (254). It can be used in the diagnosis and monitoring of diabetes, but is reserved for instances where HbA1c cannot be used, such as haemoglobinopathies. HbA1c is preferred due to its greater stability and reliability as a diabetes marker (253). It has been selected for use in this study due to its ability to better represent short-term glycaemic handling. This is of particular value in assessing AI patients who have crossed over between treatments. As these individuals will have only been on the new medication for a short period of time, fructosamine is superior to HbA1c in offering a better resolution as more half-lives will have passed before reassessment. This will mean that fructosamine is more likely to have adapted to its new baseline after a change in treatment compared to HbA1c. Fructosamine has not however been used in other AI related studies and is therefore unproven.

Whilst many of the glycaemic markers used in this study, with the exception of HbA1c, do not demonstrate a track history of detecting changes in AI populations, this is not an obstacle against their use. Although the default position must be that these markers do not have the sensitivity to detect a change, they are all proven in other biological fields. Taken together, this means that an absence of a detectable difference in any of these parameters may not constitute true evidence of "no difference". A detectable difference, does however maintain significance and must be interpreted accordingly.

5.2 Hypotheses and aims

5.2.1 Hypotheses

1- All glycaemic markers will be comparable between Groups A-C

2- Group D may show changes in markers of insulin sensitivity.

3.2.2 Aims

To compare markers of glycaemic handling between patients prescribed hydrocortisone and prednisolone replacement regimens in AI, as well as with healthy volunteers and patient receiving high doses of glucocorticoids.

5.3 Results

5.3.1 Glycaemic markers

As not all Group D participants were fasting during the study visits, it is not possible to compare their glucose, insulin and c-peptide values to Groups A-C. By extension, their HOMA indices cannot be calculated.

Fructosamine concentration were significantly lower in the hydrocortisone group and prednisolone group compared to the healthy volunteers and high dose groups, 234.2 (±16.5) and 239.9 (±19.5) mmol/L compared to 256.2 (±18.3) and 265.40 (±17.4) mmol/L (P=0.0008) respectively (Figure 5.1). There were no significant differences in HbA1c and fasting glucose (Table 5.1).

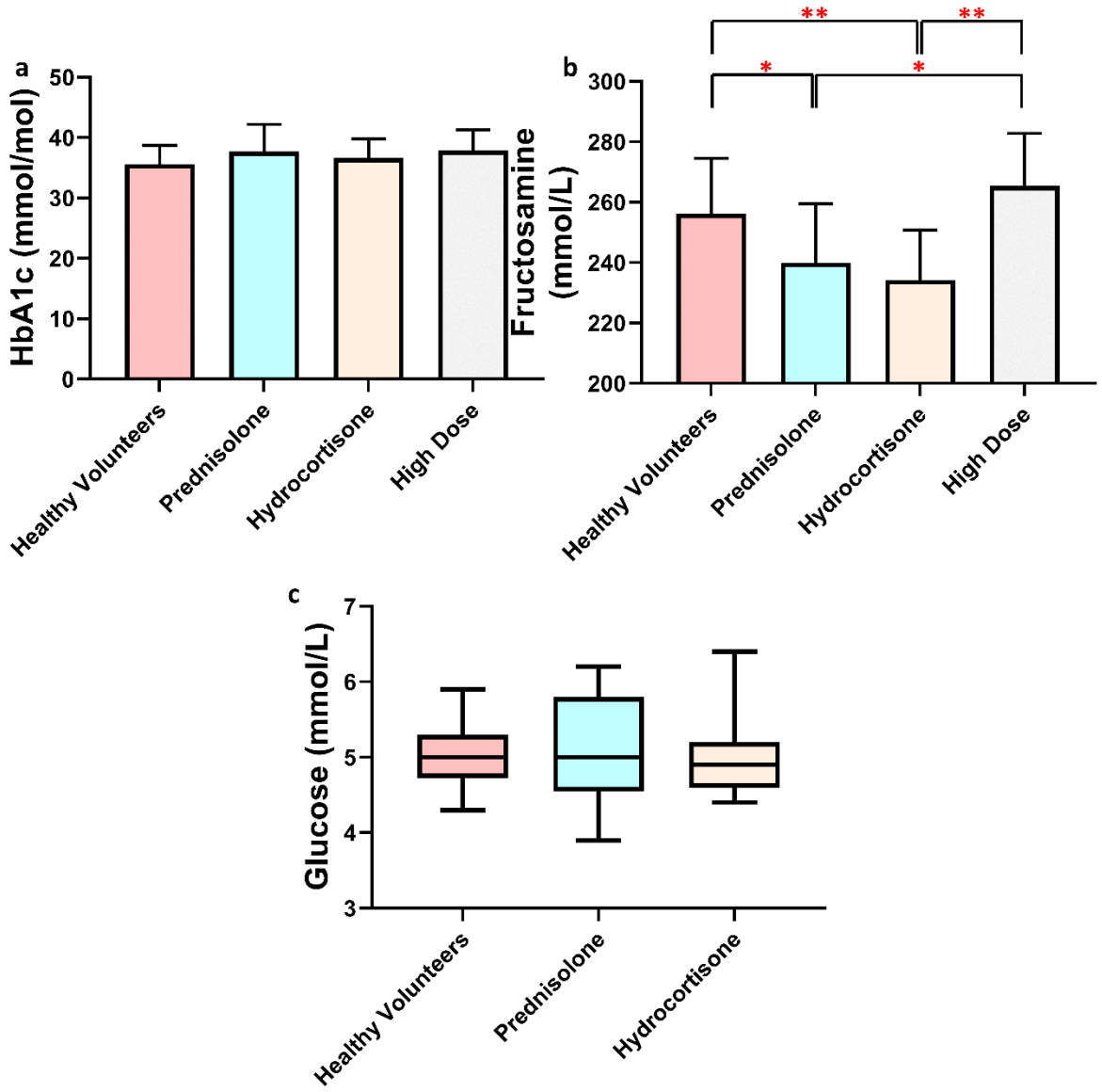

Figure 5.1- Differences between groups in HbA1c (a), fructosamine (b), and fasting glucose (c). Data is given as median, quartiles and range in (c). Data is given as mean + SD in (a), and (b). Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=19; high dose n=9. * denotes P<0.05; ** denotes P<0.01

Measurand	Group A Healthy Volunteers	Group B Prednisolone	Group C Hydrocortisone	Group D High Dose	Significance
HbA1c (mmol/mol)	35.6 (±3.1)	37.7 (±4.5)	36.6 (±3.1)	37.9 (±3.4)	P=0.24
Fructosamine (mmol/L)	256.2 (±18.3)	239.9 (±19.5)	234.2 (±16.5)	265.4 (±17.4)	P=0.0008*
Fasting Glucose (mmol/L)	5.0 (0.5)	5.0 (1.2)	4.9 (0.6)	*5.2 (1.3)*	#P=0.62
Insulin (mIU/L)	4.9 (1.5)	5.4 (3.2)	7.3 (3.5)	*7.9 (4.8)*	#P=0.039*
C-Peptide (mIU/L)	436 (142)	513 (254)	570 (255.5)	*622 (393)*	#P=0.025*
HOMA-%β	59.7 (37.6)	67.3 (48.1)	92.5 (40.7)	-	#P=0.027*
HOMA-IR	1.11 (0.35)	1.22 (1.08)	1.66 (0.84)	-	#P=0.073

Table 5.1- Data from biochemical glycaemic markers for Groups A-D. Data is reported as mean (±SD) for parametric data and median (IQR) for non-parametric data. Results in italics are non-fasting and are therefore not comparable to Groups A-C. HOMA indices are not reported, as non-fasting blood samples were used. #- denotes Kruskal-Wallis analysis of 3 groups only; excluding Group D. Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=19; high dose n=9. * denotes significant P-values.

Insulin, c-peptide concentrations and HOMA-%β were significantly elevated in the hydrocortisone group compared to the healthy volunteers (Figure 5.2). HOMA-IR was also numerically elevated in the hydrocortisone group (P=0.073).

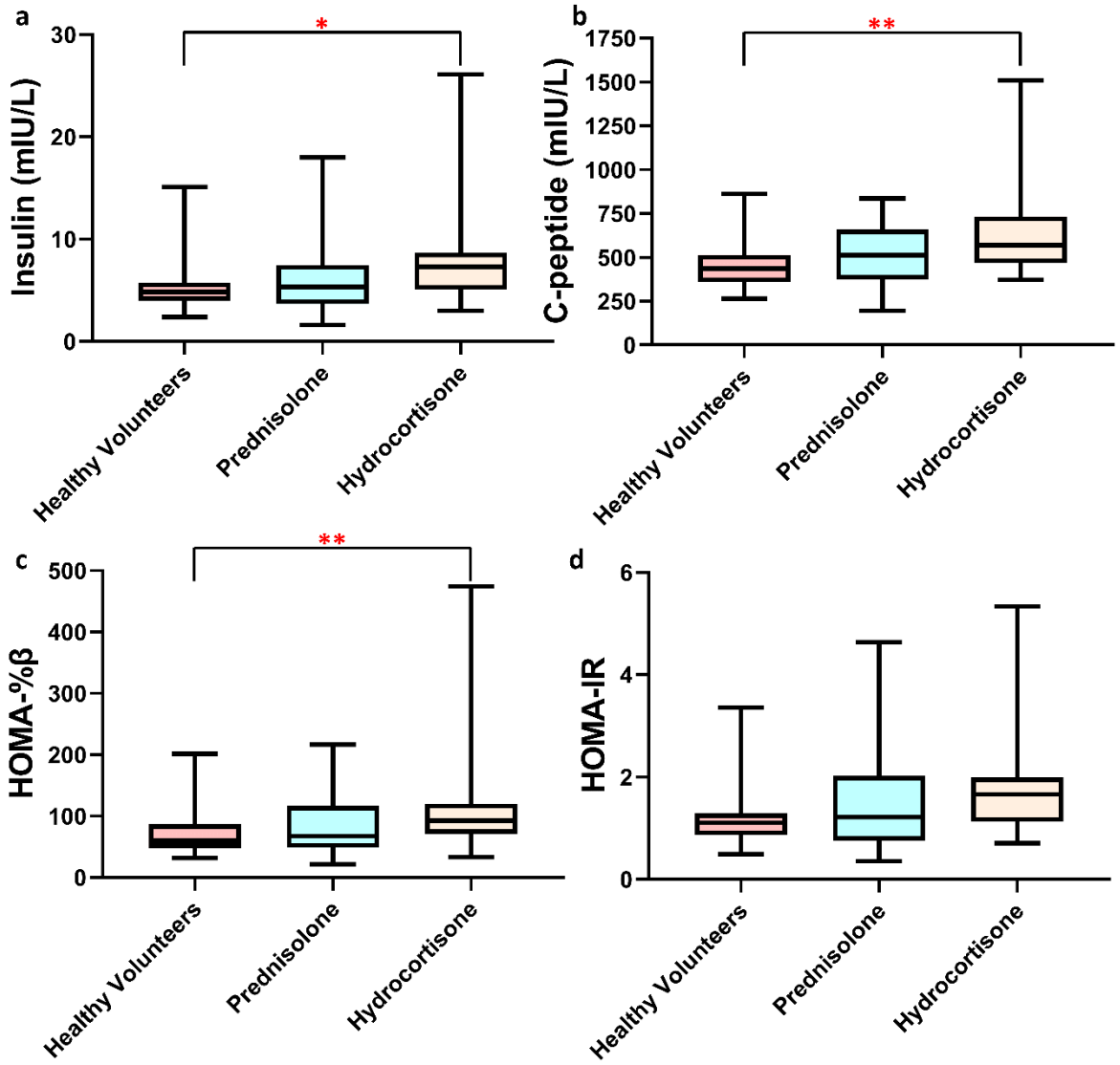

Figure 5.2- Differences between groups in insulin (a), c-peptide (b), homeostasis model assessment (HOMA)-%β (c) and HOMA-IR (d). Data is given as median, quartiles and range. Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=19. * denotes P<0.04; ** denotes P<0.03

Correlation analysis showed a significant association between insulin levels and prednisolone dose, and a trend towards association for insulin levels and hydrocortisone dose; P=0.04 and r=-0.34 and P=0.09, r= 0.13 (Figure 5.3). The correlation was negative in the case of prednisolone dose. No significant association was seen with medication dose and glucose or c-peptide. Prednisolone levels or cortisol, in the case of Group C, did not correlate with these glycaemic markers.

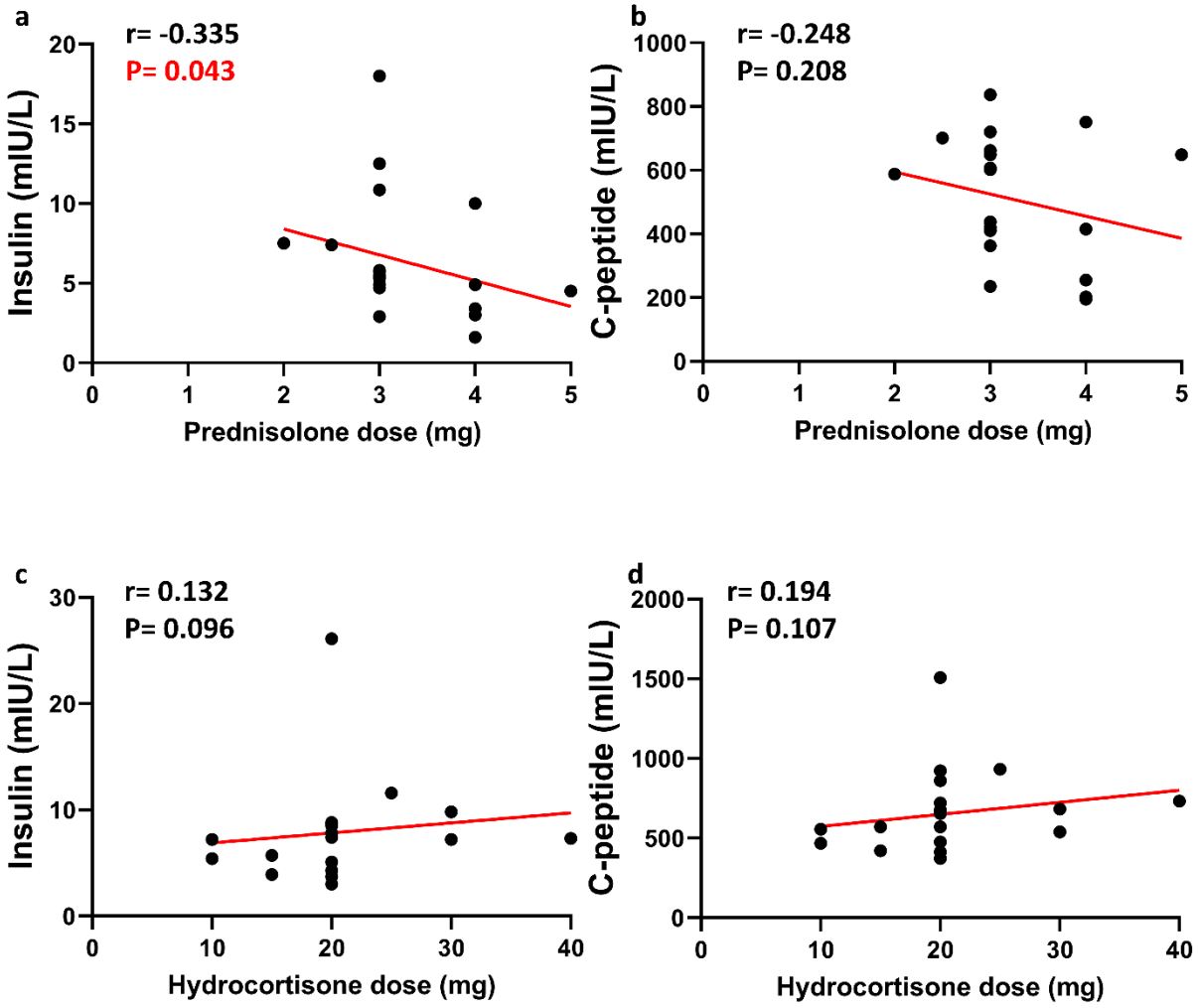

Figure 5.3- Scatterplot of prednisolone dose versus insulin (a) and c-peptide (b); hydrocortisone dose versus insulin (c) and c-peptide (d). Least squares regression line fitted. Pearson's correlation coefficient and P-values are reported. (a) and (b) n=20; (c) and (d) n=19. A significant negative moderate correlation was observed between prednisolone dose and insulin concentration.

A further correlation analysis was performed between uOC, cOC, OC indices and insulin, c-peptide, glucose and HOMA indices for Groups A-C. The correlation matrices did not yield any significant associations other than uOC versus HOMA-%β for healthy volunteers and uOC:cOC ratio versus HOMA-IR for the prednisolone group (Figure 5.4)

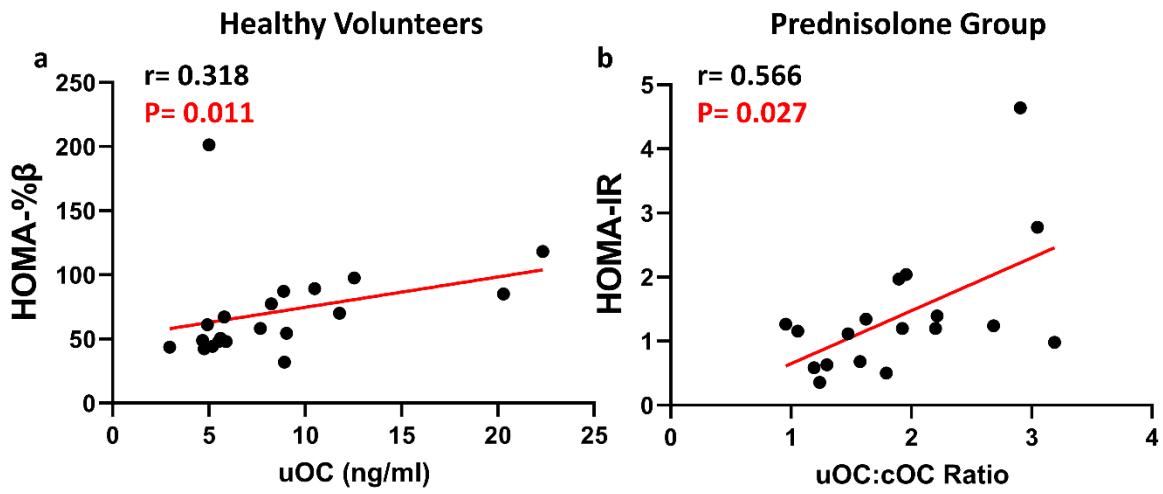

Figure 5.4- Scatterplot of undercarboxylated osteocalcin (uOC) versus homeostasis model assessment (HOMA)-%β in healthy volunteers (a) and uOC: carboxylated osteocalcin (cOC) ratio versus HOMA-IR in the prednisolone group (b). Least squares regression line fitted. Pearson's correlation coefficient and p-values are reported. Healthy volunteers n=20; prednisolone n=18. A significant positive moderate correlation was observed in both graphs.

5.3.2 Data from crossover analysis

Crossover analysis did not show any significant change in any of the glycaemic parameters measured in the 9 included patients (Figure 5.5). Only 5 pairs of fructosamine values were compared, due to missing data.

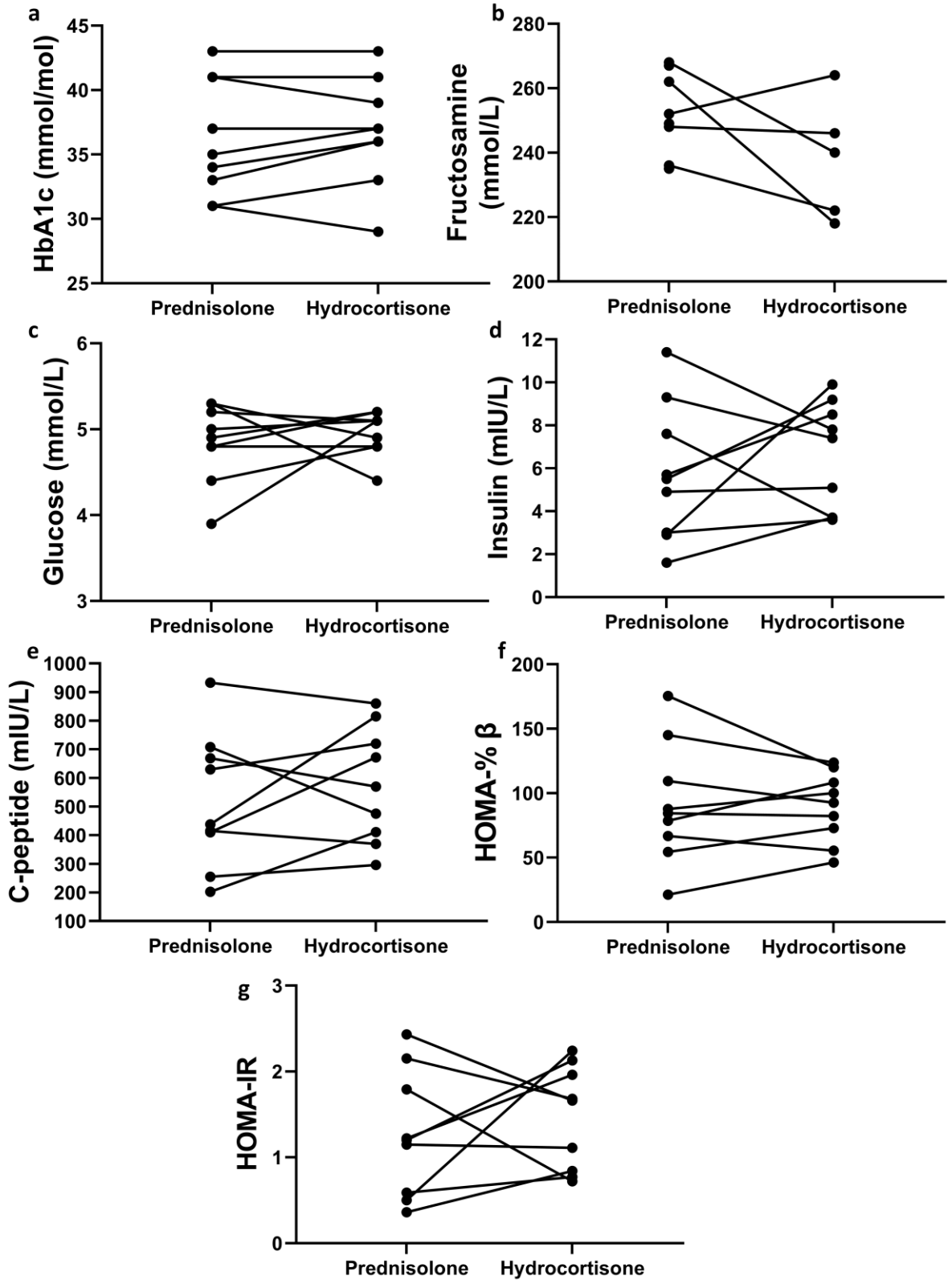

Figure 5.5- Changes in individual levels of HbA1c (a), fructosamine (b), glucose (c), insulin (d), c-peptide (e), homeostasis model assessment (HOMA)-%β (f) and HOMA-IR (g) between the same individuals on prednisolone and hydrocortisone. N=9. No significant differences between groups were seen.

5.4 Discussion

Whilst there was no difference in HbA1c and fasting glucose, the prednisolone and hydrocortisone groups showed significantly lower concentrations of fructosamine than the healthy volunteers and the high dose group. These findings are remarkably discordant. The difference between Groups A and D correspond with expectations. Although not significant, individuals receiving weekly methyl-prednisolone are likely to have greater insulin resistance that manifests as deterioration of short-term markers such as fructosamine. It also follows that HbA1c may not have deteriorated sufficiently to show a significant change due to short a sampling interval, but is certainly still higher than in the healthy volunteers. It is however incongruous that as expected, the HbA1cs of Groups B and C are higher than that of the healthy volunteers (albeit without achieving significance), but the fructosamine levels are in fact significantly lower. There is no clear explanation for this. There is evidence of glucocorticoids increasing protein turnover(255,256). It is possible that chronic subclinical glucocorticoid excess has a different effect on protein glycation than acute mega-excess as seen in Group D. Chronic exposure may influence protein glycation differently due to glucocorticoid mediated tissue protein catabolism. It is possible that excess free amino acids have greater predilection to glycation than full proteins. Further work will be needed to elucidate this.

Of note, raised insulin levels, c-peptide levels and HOMA-%β in the hydrocortisone group compared to healthy volunteers paints a concordant picture of relative hyperinsulinaemia. In addition, the increased HOMA-IR in the hydrocortisone group trended towards significance indicating that the study may have been underpowered to detect a change in this parameter. It is noteworthy that there were no differences between hydrocortisone and prednisolone, suggesting that if there is relative excess glucocorticoid exposure in the hydrocortisone group, glycaemic measures are not sensitive enough to pick this up. Unfortunately, the high dose glucocorticoid group did not all observe a fasting period prior to their study visits due to the impracticality of asking individuals to fast until an uncertain time

in the afternoon. The absence of data from this group is detrimental to interpretation of the results. It is expected that Group D would have shown an increased serum concentration of insulin and c-peptide with concordant elevations of the HOMA indices. This would have gone towards demonstrating the dose dependant effect of glucocorticoids worsening glycaemic handling.

Interestingly, there is further discordance in the correlation analyses. Prednisolone and cortisol levels in Groups B and C respectively, do not show an association with insulin secretion or HOMA metrics. There is however association with the medication dosage. Similar findings were observed in the analysis of bone markers. This would continue to suggest that 2-hour post administration drug levels are not representative of total glucocorticoid exposure of systemic effect. This corresponds with 2-hour drug levels not having any clinical utility (49,90). Further, insulin levels showed a negative correlation with prednisolone dose and tended towards a significant positive correlation with hydrocortisone dose. This is unexpected, as both should have demonstrated a positive correlation. There may not have been sufficient numbers of patients to provide power to detect the association in the hydrocortisone group. The observation of a negative association with prednisolone dose could possibly be to do with the manner of dose titration. As the majority of patients require 3- 4 mg of prednisolone, these individuals are more likely to be over-replaced than the individuals on 2, 2.5 or 5 mg. Those on less common doses, will have been titrated more strictly and with greater attention and concern according to 8-hour prednisolone levels. It therefore follows that the individual receiving 5 mg, will not have the highest insulin concentration because they have already been closely monitored. Individuals receiving lower or higher than usual doses of hydrocortisone are more likely to have been titrated clinically or based on less objective hydrocortisone day curves, increasing the likelihood of a positive correlation.

C-peptide and insulin are produced from proinsulin in an equimolar fashion. It would therefore be expected that any analyte which shows a correlation with insulin, should also show correlation with

c-peptide, but this was not the case in this study. Although insulin is less stable than c-peptide, stability is unlikely to be the source of the problem as samples were collected in the present study in near perfect conditions, and frozen quickly. It is more likely that the differences seen are due to the mode of extraction and excretion of both molecules. Insulin is extracted by the liver and cleared, whereas c-peptide is renally excreted (257). The net effect is that insulin is excreted at a faster rate than c-peptide (258). This explains why insulin showed better association with glucocorticoid dosing.

Given evidence in the literature of uOC influencing and enhancing insulin secretion and sensitivity, analysis included assessment of association between osteocalcin and insulin indices. Multiple correlation analysis did not reveal any associations other than a moderate positive correlation between uOC and HOMA-%β in healthy volunteers and a moderate positive correlation between uOC:cOC ratio and HOMA-IR. The former is in keeping with expectations from the literature, namely that greater levels of uOC cause increased β-cell insulin secretion. The latter is more difficult to interpret. Increasing uOC:cOC ratio should cause reductions in HOMA-IR, but the opposite was observed. This is in the context that earlier finding show that higher prednisolone doses are associated with lower uOC:cOC ratios, but should actually cause worsening insulin resistance as per glucocorticoid class effects. These findings must be cautiously considered. The multiple correlation analysis was an exploratory enterprise based on the current literature, but in the absence of a multiple comparison correction, it is possible that this is a type 1 error.

The analysis of glycaemic outcomes did not show any significant difference between individuals receiving hydrocortisone and prednisolone. This is further corroborated by the crossover analysis, which did not show any differences when individuals switched from one therapy to another.

It is difficult to consider these results in the context of previous studies. As the literature is heterogenous, there is no proper consensus of the expected findings in comparing a cohort of patients receiving hydrocortisone to healthy volunteers. These findings do not agree with a cross-sectional case-control study assessing 76 healthy volunteers and AI patients (203). The result of the aforementioned study found an increased HbA1c and unchanged HOMA indices. There are very few other papers that prospectively compare these indices between healthy individuals and patients with AI, and none of these published completely concordant findings (199). There are currently no studies directly comparing low dose prednisolone as used at ICHNT, with hydrocortisone regimens or healthy volunteers.

The outcome of this analysis shows signals that hydrocortisone replacement may cause minor changes to glycaemic profiles compared to healthy volunteers. There appears to be a propensity for hyperinsulinaemia in the context of normoglycaemia with a trend towards increased insulin resistance. As with the previous analysis there is no clear difference between the two treatments for AI.

Chapter 6: The Effects of Different Glucocorticoid Regimens on Infection Rates and Immunology Profiles

6.1 Introduction

Relatively little in-vivo research has been completed in AI patients to characterise haematological and immunological differences. Although there is an abundance of basic science research in this area, very few of these studies are clinically applicable.

Infectious diseases are a significant problem in patients with AI. A Swedish registry population cohort study demonstrated that individuals with PAI who develop an infection have an SMR of 5.9 (32). Another Swedish study further characterised the infection burden, finding that pneumonia accounted for 66% of infections and that men and women had a 6.6-fold and 5.6-fold increased risk of death respectively, when admissions are complicated by infection (31). Data on SAI patients is more scarce as studies tend to focus on growth hormone or hypopituitarism rather than SAI. A large global multi-centre study ascertained that the SMR of death secondary to infection in growth hormone replaced patients was 4.97 (259). They found that 82.6% of the recorded deaths due to infection were complicated by SAI. Further, the presence of ACTH deficiency in this cohort increased the risk of death by infection by 1.6 times.

Recent UK-centric data from 1987 to 2017 including PAI and SAI patients demonstrated an increased risk of death from infection, with a hazard ratio of 4 (196). Patients with PAI were noted to be at greater risk than patients with SAI.

The most detailed information is derived from a study of 390 PAI patients compared to 1933 controls (111). Compared to the control group, infection rates were significantly elevated in the PAI cohort by a factor of 1.5. The adjusted number of prescriptions for antiviral and antifungal medication in PAI

patients were nearly 2 times greater than in controls. Antibiotic prescriptions were 1.5x greater, in keeping with other available data (260). The risk of admission secondary to an infection was in keeping with figures reported by other studies, at approximately a 5-fold increase. The commonest infections were urinary tract infections and pneumonia. These findings are echoed by more recent studies (261).

The literature to date intimates that AI is linked to increased bacterial infections, and possibly increased viral and fungal infections, by way of tracking increased numbers of prescriptions. For viral and fungal infection, hospital admissions are not a good method of estimating incidence as they seldom are a reason for admission. The pathophysiology of these observations is less clear.

A study of 42 patients with PAI drew conclusions from a comparison with 58 matched healthy controls (262). In this cross-sectional study, a group assessed neutrophil and NK cell function and phenotype. Although the study reported normal neutrophil function, they noticed a trend towards reduced phagocytic activity from opsonisation assays. It was likely that the study was underpowered, but it provided signals towards neutrophil dysfunction which might explain the propensity for severe bacterial infections in PAI that lead to hospital admissions. More significantly, NK cells in PAI demonstrated impaired NK induced cellular cytotoxicity. Further NK cells in PAI were noted to express activating receptors NKG2d, NKp30 and NKp46 with lower frequency. Taken together, this would explain observations of reduced viral immunity in PAI patients.

By obtaining PBMC samples from 53 patients with PAI, and comparing them to 75 matched controls, a Norwegian study has indirectly demonstrated PBMC dysfunction. CXCL10 is a chemokine that is elevated in patients with PAI. CXCL10 is implicated in adaptive immunity where it provides chemoattractant cue for T-cells, and is secreted by PBMCs in response to interferons, which are key to viral defence. The group ultimately showed that the elevated CXCL10 levels were likely to be

secreted by damaged adrenal tissue, but in the process identified that PBMCs from PAI patients were less responsive to interferons. This reduced sensitivity could also explain impaired viral immunity.

The DREAM study has previously been reviewed in depth (108). It is a study of significance which showed normalisation of classical monocytes, non-classical monocytes and NK cells in response to changing treatment regimen to MR-HC. These three populations are important in innate bacterial defence and viral immunity. The study also linked reductions in viral illnesses to the normalisation of these profiles. Together, this paints a compelling picture associating imperfect glucocorticoid replacement with white cell dysfunction and consequent viral illness. The success of the DREAM study has informed the present study, in the use of the GNCQ to tabulate infection rates in the study population in the absence of any other validated questionnaires being used in glucocorticoid literature. The GNCQ is a validated questionnaire for the collection of infection data itself (263,264). Further it has prompted us to quantify the same cell populations in order to understand whether these findings can be replicated with prednisolone or hydrocortisone therapy.

The DREAM study raises the question whether it is more physiological replacement with MR-HC or the reduced glucocorticoid exposure evidenced by the 20% reduction in cortisol AUC that is responsible for the benefits seen. Unfortunately, this question has yet to be answered by an appropriately designed study. Corticosteroids are obligatory immunomodulators that are not well characterised at present (3,265). Whilst there is overwhelming real-world clinical data that very high doses are anti-inflammatory and immunosuppressive, it is not understood how a mild excess truly affect white cells. Further, there is underappreciation that basal levels of glucocorticoids are essential for immune function and antibody production (266). Therefore, under-replacement is also likely to provoke immune compromise. The lack of research into the interaction of CLOCK genes with timing of glucocorticoids and immune function is another obstacle to our understanding of the complicated interplay of all these factors.

6.2 Hypotheses and aims

6.2.1 Hypotheses

1- Infection rates will be higher in the AI groups compared to the healthy volunteers

2- Infection rates will be comparable between patients receiving prednisolone and hydrocortisone

3- White cell populations will be comparable between Groups A-C and elevated in Group D

4- NK cell and monocyte populations will be altered between AI groups and healthy volunteers

5- NK cell and monocyte populations will be comparable between AI groups

6.2.2 Aims

To compare infection rates and white cell populations between patients taking hydrocortisone and prednisolone replacement regimens in AI, as well as with healthy volunteers and patient receiving high doses of glucocorticoids.

6.3 Results

6.3.1 Infection rates

Data collected using the GNCQ did not demonstrate any significant differences in infection rates between Groups A-D (Table 6.1).

	Group A Healthy Volunteers					Group B Prednisolone					Group C Hydrocortisone					Group D High Dose					P-value
Frequency	0	1	2	3	>3	0	1	2	3	>3	0	1	2	3	>3	0	1	2	3	>3	
URTI	10	5	4	1		10	3	5		2	12	4	1	2	1	8	1				P=0.38
URTI (Weighted)	1.25 (1.5)					1.25 (1.5)					0.5 (1.25)					0 (0.5)					P=0.40
Pneumonia	20					20					19					9					N/A
Pneumonia (Weighted)	0.5 (0)					0.5 (0)					0.5 (0)					0.5 (0)					N/A
GI infection	19	1				18	1				17	2	1			8	1				P=0.79
GI infection (Weighted)	0.5 (0)					0.5 (0)					0.5 (0)					0.5 (0)					P=0.61
Skin Infection	18		1	1		18	2				17	1			1	8	1				P=0.65
Skin Infection (Weighted)	0.5 (0)					0.5 (0)					0.5 (0)					0.5 (0)					P=0.99
Boils	20					20					19	1				9					P=0.47
Boils (Weighted)	0.5 (0)					0.5 (0)					0.5 (0)					0.5 (0)					P=0.50
UTI	20					16	4				18	2				7	2				P=0.17
UTI (Weighted)	0.5 (0)					0.5 (0)					0.5 (0)					0.5 (0.75)					P=0.12
Flu	16	4				15	3	1		1	15	4			1	7	1	v		1	P=0.85
Flu (Weighted)	0.5 (0)					0.5 (0.375)					0.5 (0.375)					0.5 (0.75)					P=0.93

Table 6.1- Weighted German National Cohort Questionnaire (GNCQ) results and frequency of infections in each group. Categorical data is presented in blue cells. Median weighted scoring (IQR) as per GNCQ protocol is presented in the white cells. Chi-squared test performed for categorical data and Kruskal Wallis for weighted scores. Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=20; high dose n=9. No significance was detected. URTI- upper respiratory tract infection; GI- gastrointestinal; UTI- urinary tract infection.

6.3.1 Data from white cell differentials

WBC was significantly elevated in the hydrocortisone group and high dose glucocorticoid groups compared to the healthy volunteers (Figure 6.1). All glucocorticoid groups showed significantly higher levels of neutrophils than seen in the healthy volunteer cohort. There were no significant differences between groups in lymphocyte count (Table 6.2). Monocyte counts were highest in the hydrocortisone group and lowest in the high dose group. They were significantly higher in the hydrocortisone group compared to the healthy volunteers. Eosinophils were also lowest in the high dose group and were noted to be significantly lower than the AI groups.

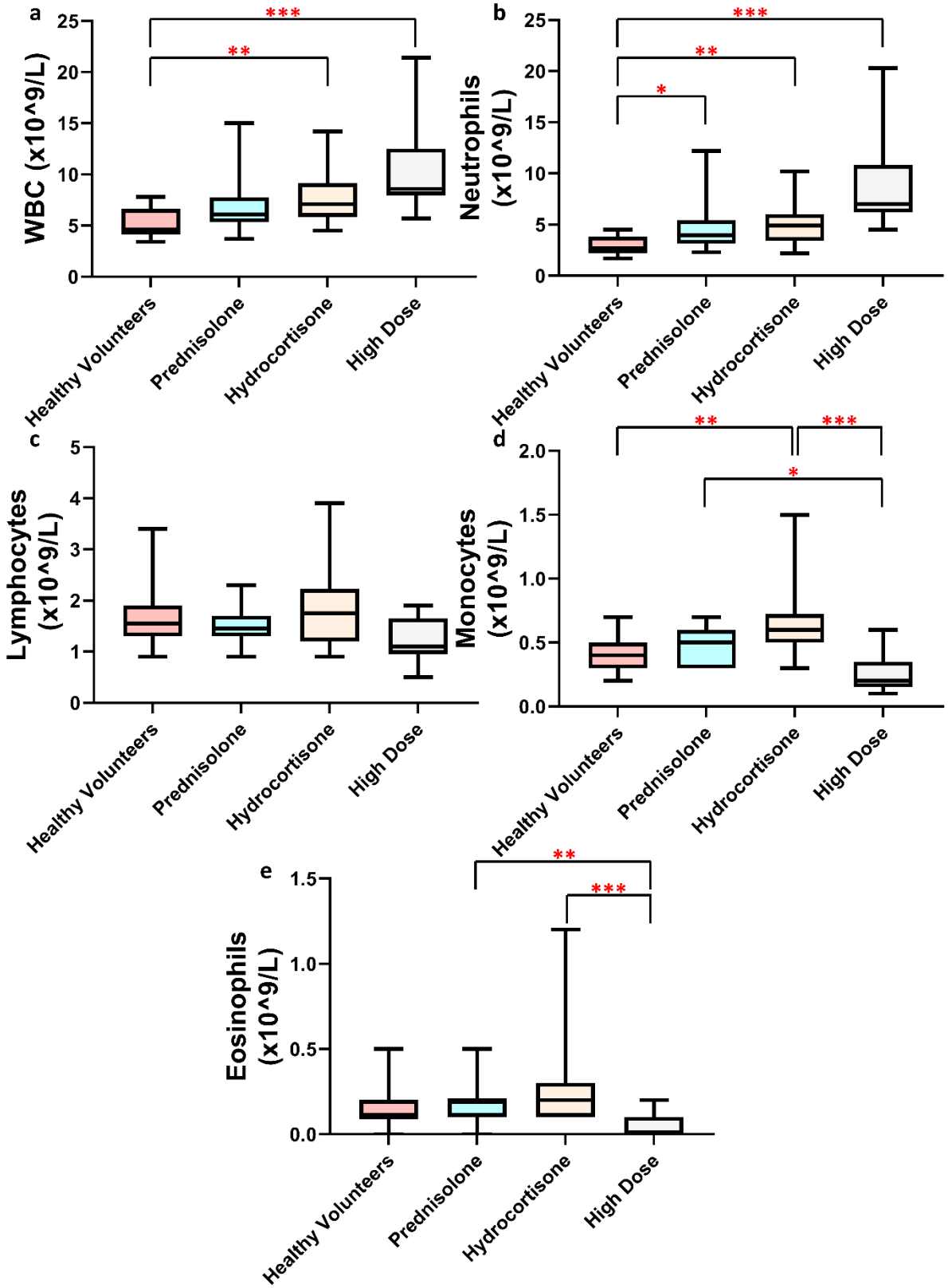

Figure 6.1- Differences between groups in white blood cell count (WBC) (a), neutrophils (b), lymphocytes (c), monocytes (d) and eosinophils (e). Data is given as median, quartiles and range. Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=19; high dose n=9. * denotes significance P<0.03 ; ** denotes significance P<0.005; *** denotes significance P<0.0001

Measurand	Group A Healthy Volunteers	Group B Prednisolone	Group C Hydrocortisone	Group D High Dose	Significance
WBC ($x10^9$/L)	4.6 (2.3)	6.1 (1.8)	7.1 (3.0)	8.6 (1.3)	P<0.0001*
Neutrophils ($x10^9$/L)	2.7 (1.4)	4.0 (1.7)	4.9 (2.3)	7.0 (1.3)	P<0.0001*
Lymphocytes ($x10^9$/L)	1.6 (0.6)	1.5 (0.4)	1.8 (1.0)	1.1 (0.5)	P=0.13
Monocytes ($x10^9$/L)	0.4 (0.2)	0.5 (0.3)	0.6 (0.2)	0.2 (0.1)	P<0.0001*
Eosinophils ($x10^9$/L)	0.1 (0.1)	0.2 (0.1)	0.2 (0.2)	0 (0)	P=0.0002*

Table 6.2- Results of measured white cell differentials. All data is reported as median (IQR). Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=19; high dose n=9. Significance P-values from Kruskal Wallis analysis.

6.3.2 Data from flow cytometry analysis

There was significant suppression of human leukocyte antigen (HLA)-DR expressing monocytes in the high dose glucocorticoid group with a mean (±SD) of 39.4% (±22.0) compared to 71.3% (±13.5) and 69.5% (±9.5) in the hydrocortisone and healthy volunteer groups; P=0.003 and P=0.002 respectively (Figure 6.2 and Table 6.3). There were otherwise no significant differences between monocyte and NK cell populations (Figure 6.3). Analysis was complicated by reduced n-numbers, which are reported in Table 6.3.

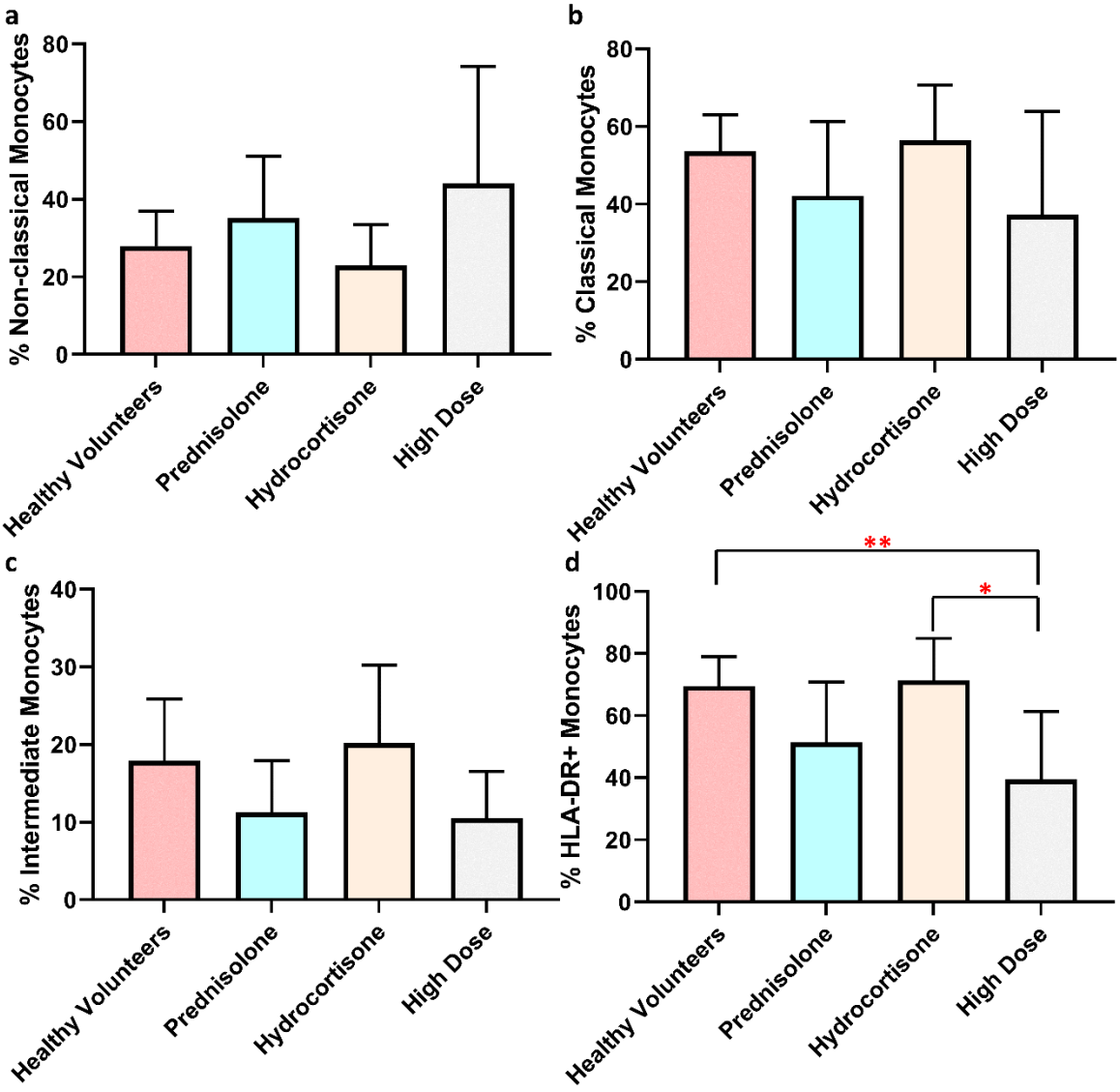

Figure 6.2- Differences in monocyte populations between groups in % non-classical monocytes (a), % classical monocytes (b), % intermediate monocytes (c) and % human leukocyte antigen (HLA)-DR+ monocytes (d). Data is given as mean + SD. Healthy volunteers n=14; prednisolone n=5; hydrocortisone n=7; high dose n=6. * denotes P<0.004; **denotes P<0.002

Population	Group A Healthy Volunteers	Group B Prednisolone	Group C Hydrocortisone	Group D High Dose	Significance
CD14-/CD16+ Non-classical monocytes (% of monocyte)	27.9 (±9.0) n=14	35.2 (±15.9) n=5	22.9 (±10.6) n=7	44.0 (±30.2) n=6	P=0.19
CD14+/CD16- Classical monocytes (% of monocytes)	53.6 (±9.4) n=14	42.2 (±19.1) n=5	56.5 (±14.3) n=7	37.2 (±26.6) n=6	P=0.11
CD14+/CD16+ Intermediate monocytes (% of monocytes)	17.9 (±7.9) n=14	11.3 (±6.6) n=5	20.2 (±10.0) n=7	10.5 (±6.1) n=6	P=0.08
HLA DR+ cells (% of monocytes)	69.5 (±9.5) n=14	51.4 (±19.6) n=5	71.3 (±13.5) n=7	39.4 (±22.0) n=6	P=0.0008*
CD56+ cells (% of lymphocytes)	5.1 (5.7) n=18	6.3 (4.5) n=8	6.9 (3.1) n=10	21.2 (13.6) n=6	P=0.08
$CD56^{Bright}$ cells (% of CD56+ cells)	6.9 (8.9) n=18	11.1 (8.9) n=8	4.8 (3.5) n=10	2.3 (4.0) n=6	P=0.08
$CD56^{Dim}$ cells (% of CD56+ cells)	90.1 (7.6) n=18	87.5 (8.5) n=8	91.1 (5.5) n=10	96.7 (5.1) n=6	P=0.10
CD16+ cells (% of CD56+ cells)	91.6 (8.3) n=18	89.6 (6.3) n=8	93.3 (6.4) n=10	92.1 (5.5) n=6	P=0.23

Table 6.3- Distribution of monocyte and natural killer (NK) cell populations ascertained by flow cytometry analysis for Groups A-D. Data is reported as mean (±SD) for parametric data and median (IQR) for non-parametric data. N numbers are reported. * denotes significant P-values.

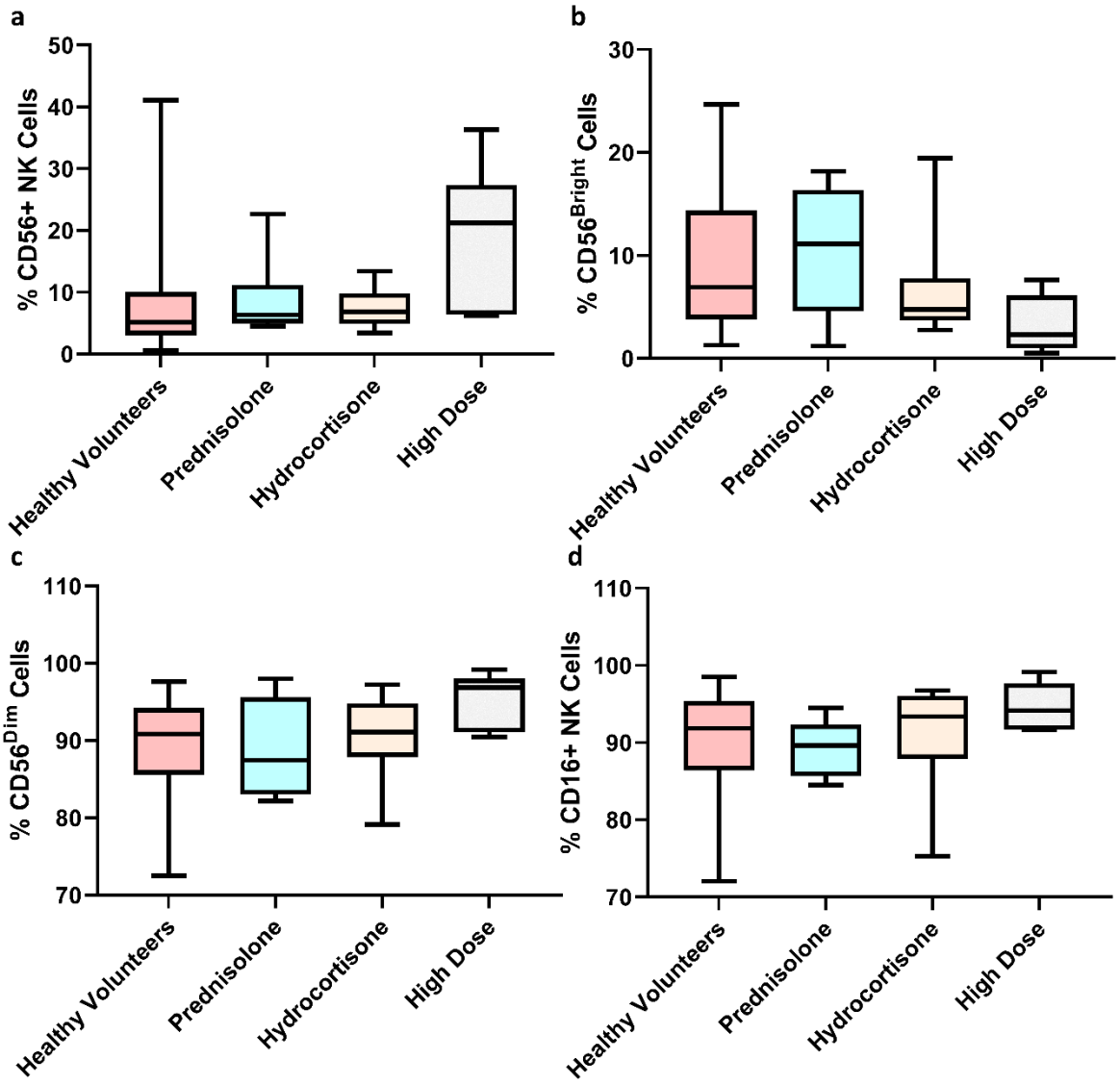

Figure 6.3- Differences in natural killer (NK) cell populations between groups in % CD56+ NK cells (a), %CD56^{Bright} NK cells (b), % CD56^{Dim} NK cells (c) and % CD16+ NK cells (d). Data is given as median, quartiles and range. . Healthy volunteers n=18; prednisolone n=8; hydrocortisone n=10; high dose n=6. No significant differences were detected.

6.3.3 Data from crossover analysis

GNCQ data could not be analysed as the questionnaire assesses the frequency of infections over the past 6 months. Individuals in this study, who had crossed over treatments, were reassessed after 4 months. Further, only 3 pairs of flow cytometry data were available for crossover analysis. Analysis was therefore not performed.

Sufficient data was available for assessment of the FBC differentials (Figure 6.4). No significant differences were detected as patients changed between treatments.

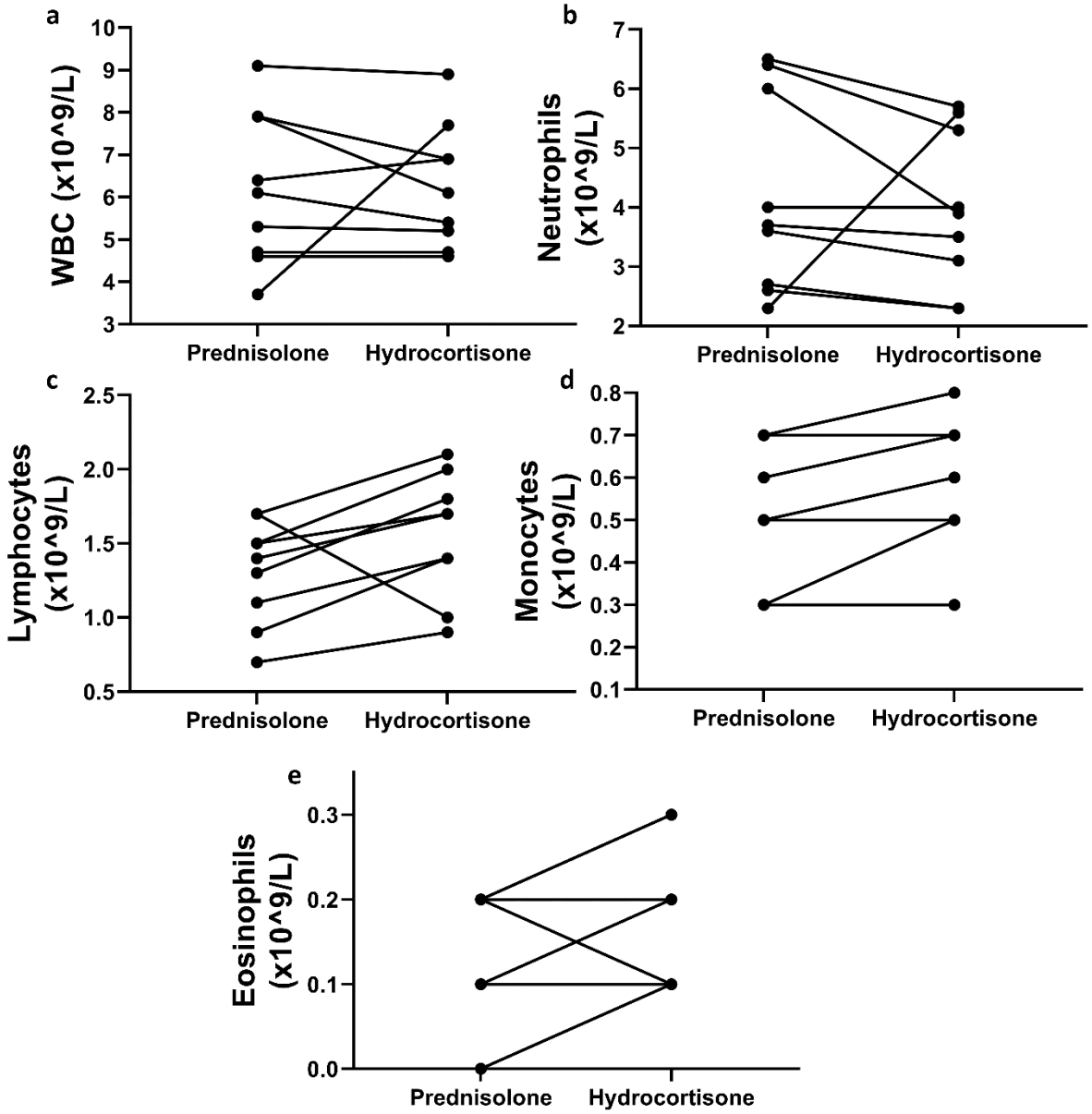

Figure 6.4- Changes in individual levels of white blood cell count (WBC) (a), neutrophils (b), lymphocytes (c), monocytes (d) and eosinophils (e). N=9. No significant differences between groups were seen.

6.4 Discussion

Analysis of the rates of infection and the differences in immune cell populations has painted a complicated picture. There is clearly no evidence of differences in infection rates between the different groups. The results from the white cell differentials suggest over-replacement in the hydrocortisone, and to a lesser degree prednisolone, cohorts. As expected, the high dose glucocorticoid group had markedly elevated WBC and neutrophil counts compared to healthy volunteers. The patients receiving hydrocortisone demonstrated the same picture, whilst the prednisolone patients only had significantly elevated neutrophils. This implies a sliding scale, where high doses of glucocorticoid cause the biggest effect followed by hydrocortisone and then prednisolone.

Increased white cells in individuals receiving glucocorticoids is due to the well documented effect of glucocorticoids inducing neutrophilia. In the acute setting, this phenomenon is due to demargination (267). Pools of marginated neutrophils have been well characterised to exist in the bone marrow, spleen and liver (268). Glucocorticoids have been shown to reduce the expression of cell adhesion molecules by neutrophils permitting them to release from margination pools causing a sudden rise in levels (269). Neutrophil counts rise as soon as 2 hours after ingestion of prednisolone 5 mg and IV infusion of 25 mg hydrocortisone (270). Unfortunately, evidence has not been generated for doses less than 5 mg of prednisolone or for oral doses of hydrocortisone in the order of 10 mg. It is however possible that upon ingestion of their morning regimens, there is a mild demargination effect in these patients that is being detected by the study especially as bloods were collected 2 hours after tablet administration. If so, as the magnitude of demargination response is proportional to the amount of corticosteroid ingested or infused, this would indicate that oral hydrocortisone in the morning is causing greater exposure and by extension, demargination than prednisolone.

Long term chronic glucocorticoid replacement may also act on neutrophil counts via a second or alternative mechanism involving granulocyte colony stimulating factor (G-CSF). A study involved 9 male volunteers who underwent a saline infusion, high dose dexamethasone infusion and low dose dexamethasone infusion in double-blind, three-way crossover trial (271). Whilst the saline infusion caused no effect, the dexamethasone infusions caused dose dependent increases in neutrophil count and G-CSF at 24 hours. Similar findings have been reported with IV-methylprednisolone causing rises in G-CSF levels at 4 hours, suggesting class effect (272). Further, G-CSF will promote long-term development and maturation of neutrophils, but continual glucocorticoid excess has the potential to further enhance and augment this process via signal transducer and activator of transcription 5 (STAT5) activation (273).

The monocyte count was noted to be significantly higher in the hydrocortisone group compared to the healthy volunteers. The high-dose group had significantly lower monocytes than the AI groups. The latter finding must be treated with caution, as there is limited scope in comparing Group D to Groups B and C. There is a visible trend forming in Figure 6.1D, whereby the replacement regimens seem to be causing rising monocyte counts, with hydrocortisone reaching significance, but anti-inflammatory doses of glucocorticoids causing suppression. It is difficult to find representation of this in the literature, as only few in-vivo studies have been performed, particularly with lower dose glucocorticoids as used in AI. Monocytopaenia is however documented in the context of oral glucocorticoids in a study that involved administration of 50 mg prednisolone twice daily to healthy volunteers (274).

A similar picture has emerged with the eosinophil data from this study. Again, there is very little literature that describes eosinophilia in the context of chronic glucocorticoid use as this area is not well researched. There is however record of falling eosinophil counts after administration of “potent” glucocorticoids, after a few hours, which reach nadir at 4 to 8 hours (275). This may be due to

glucocorticoid induced inhibition of IL-5 and granulocyte/macrophage colony stimulating factor (GM-CSF) (276,277). This in turn prevents eosinophil production and maturation in the bone marrow. Alternatively, there is evidence from rodents that cortisol may cause sequestration of eosinophils to the spleen and lymphoid tissue (278).

The flow cytometry analysis in this study was notably underpowered, with many trends forming but only one significant finding. It is clear that even with small sample numbers, high-dose glucocorticoids cause significant suppression of HLA-DR in monocytes. This is a protein that is key to adaptive immunity, being part of antigen presentation. This finding is well documented in the literature from 30 to 40 years ago (279). The trends that begin to appear are in fact more intriguing. In the case of HLA-DR, hydrocortisone is similar to the healthy volunteers, but prednisolone appears to tend towards suppression. The same pattern emerges for the classical, non-classical and intermediate monocytes, albeit in the absence of significance; prednisolone tends towards the trend set by high-dose glucocorticoids whilst hydrocortisone tends towards the healthy volunteers. On the surface, this might indicate that prednisolone is trending towards the side of over-replacement, but this is incongruent with the previous findings that it is hydrocortisone that is more likely to over-replace. The most plausible explanation is that these experiments are demonstrating a prednisolone drug-specific effect that is not in keeping with the rest of the glucocorticoid class. All of the high dose glucocorticoid patients were in receipt of IV methyl-prednisolone or oral prednisolone. As such, they are an extension of Group B. It is therefore possible that Group B and Group D were exerting a prednisolone specific effect on the different populations of monocytes, especially as the direction of the effects are continually the same, and Group D expresses the change with greater magnitude.

The NK cell analysis did not reach any significance. It appears that patients receiving high dose glucocorticoids do seem to have more NK cells (as identified by CD56+ status) as a proportion than the other 3 groups. More of these cells seem to be distributed as mature $CD56^{Dim}$ cells than maturing

$CD56^{Bright}$ cells when compared to the other groups. It is possible that high dose glucocorticoids promote maturing of NK cells, but greater sample number and better evidence of this is needed. These results are not in keeping with the DREAM study, as the CD16 expressing NK cells in the AI groups are comparable to the healthy volunteers and high dose group. Further the AI groups showed an inconsistent picture of monocyte distribution that cannot be directly compared.

It is possible that this data represents the notion that tissues react differently to different glucocorticoids. The flow cytometry data, although not achieving significance, is suggesting the converse and monocytes begin to suppress. It is possible that each glucocorticoid has preference for greater potency on specific tissues. Whilst hydrocortisone may be more potent on bone, prednisolone may be more potent on monocytes. This would be in keeping with findings that prednisolone induced gene transcription changes are not conserved, even across blood cells. For instance, the transcription changes are more potent on T-cells than monocytes (280). Given the ambiguity of these findings, more work looking at the effects of replacement regimens on haematological indices is needed.

Chapter 7: The Effects of Different Glucocorticoid Regimens on Subjective Health

7.1 Introduction

The effects of medical treatments on wellbeing are very difficult to assess. The use of questionnaires is an easy method to attempt to quantify subjective health. Prior to use and interpretation, questionnaires must be validated. The SF36 was employed in this study, due to its widespread use in Endocrine literature (153). The Addison's quality of life questionnaire (Addi-QOL) was considered for use, but decided against as it only validated in PAI cohorts, and would not therefore allow reliable comparison to the healthy volunteers and high-dose cohorts (281,282).

Prior to the SF36 being deployed, non-standardised visual analogue scales were used (62). Results were not comparable between studies and the questions were not thorough enough to examine different domains of subjective health. The SF36 is however able to provide scores in the following domains: physical functioning, role functioning (physical), role functioning (emotional), energy/fatigue, emotional wellbeing, social functioning, pain, general health and health change. The versatility of the SF36 was demonstrated in an early study. Ninety-seven patients with PAI were identified from a Norwegian register, posted a paper version of the SF36 and encouraged to respond (283). From a dataset of 79 patients, it was noted that the energy/fatigue and general health scores were significantly lower in the PAI cohort compared to previously collected normative data from healthy individuals.

Another postal, cross-sectional study of 256 patients in Germany using the SF36, showed that with the exception of the pain domain, there was significant impairment in all other domains in PAI and SAI patients compared to matched controls (284). Interestingly, patients with SAI had lower physical

functioning score than patients with PAI. The majority of patients in the study were hydrocortisone or cortisone acetate with only one individual receiving prednisolone.

Prednisolone regimens have been compared to hydrocortisone regimens using subjective health outcomes (285). A cross-sectional analysis of 427 responses from PAI and SAI patients, included 61 patients receiving prednisolone and 347 using hydrocortisone with the remainder prescribed cortisone acetate. Of note, the patients receiving prednisolone were noted to have a significantly higher blood pressure than the hydrocortisone and cortisone patients. In the absence of dosing data, this would suggest that the prednisolone patients were relatively over-replaced. It is however possible that this is a misnomer because there is a possibility of greater frequency of non-glucocorticoid related idiopathic hypertension in the prednisolone cohort due to homogeneity of the population. Later publications have suggested that the East German population may be homogenous with increased frequencies of type 1 diabetes and raised LDL compared to a heterogenous population such as that in London (151,286). Regardless, AI patients continued to show reduced global SF36 scores compared to healthy controls. In particular, prednisolone therapy was associated with significantly worse pain scores than hydrocortisone but was otherwise comparable in the other SF36 domains.

The SF36 has also shown itself to be sensitive to differences between dosing regimens (287). Considering 334 SF36 responses, individuals were grouped according to their daily prescribed amount of hydrocortisone. Whilst no differences were detected in most of the domains, role functioning (physical) and general health were noted to be significantly impaired in those taking >30 mg of hydrocortisone daily compared to <25 mg daily.

There is reasonable evidence that the SF36 is robust enough to detect subtle changes in subjective health. Its inclusion in other glucocorticoid replacement studies, provides confidence that the results from the present study will be comparable to the relevant trials of the past.

7.2 Hypotheses and aims

7.2.1 Hypotheses

1- SF36 scores will be lower in the AI groups and high-dose glucocorticoid groups compared to the healthy volunteers
2- There will be no difference in scores between AI patients receiving prednisolone or hydrocortisone

7.2.2 Aims

To compare subjective health and wellbeing between patients taking hydrocortisone and prednisolone replacement regimens in AI, as well as with healthy volunteers and patient receiving high doses of glucocorticoids.

7.3 Results

7.3.1 SF36 data

Four of the 9 SF36 domains showed a significant difference between groups (Figure 7.1). The prednisolone cohort showed a significant impairment in energy/fatigue score compared to the healthy volunteers. The mean (±SD) score was 58.2 (±21.7) and 77.3 (±10.2) respectively; P=0.022 (Table 7.1). The high dose glucocorticoid patients showed a reduced role functioning (physical) score compared to the hydrocortisone group and the healthy volunteers in particular; P= 0.035 and P=0.0056 respectively. The high dose glucocorticoid group also demonstrated reduced social functioning, to all 3 other groups. The median (IQR) score compared to the healthy volunteers was 77.3 (10.2) versus 56.3 (50.0); P=0.0003. Pain scores were significantly impaired in the hydrocortisone group compared to the healthy volunteers; P=0.023.

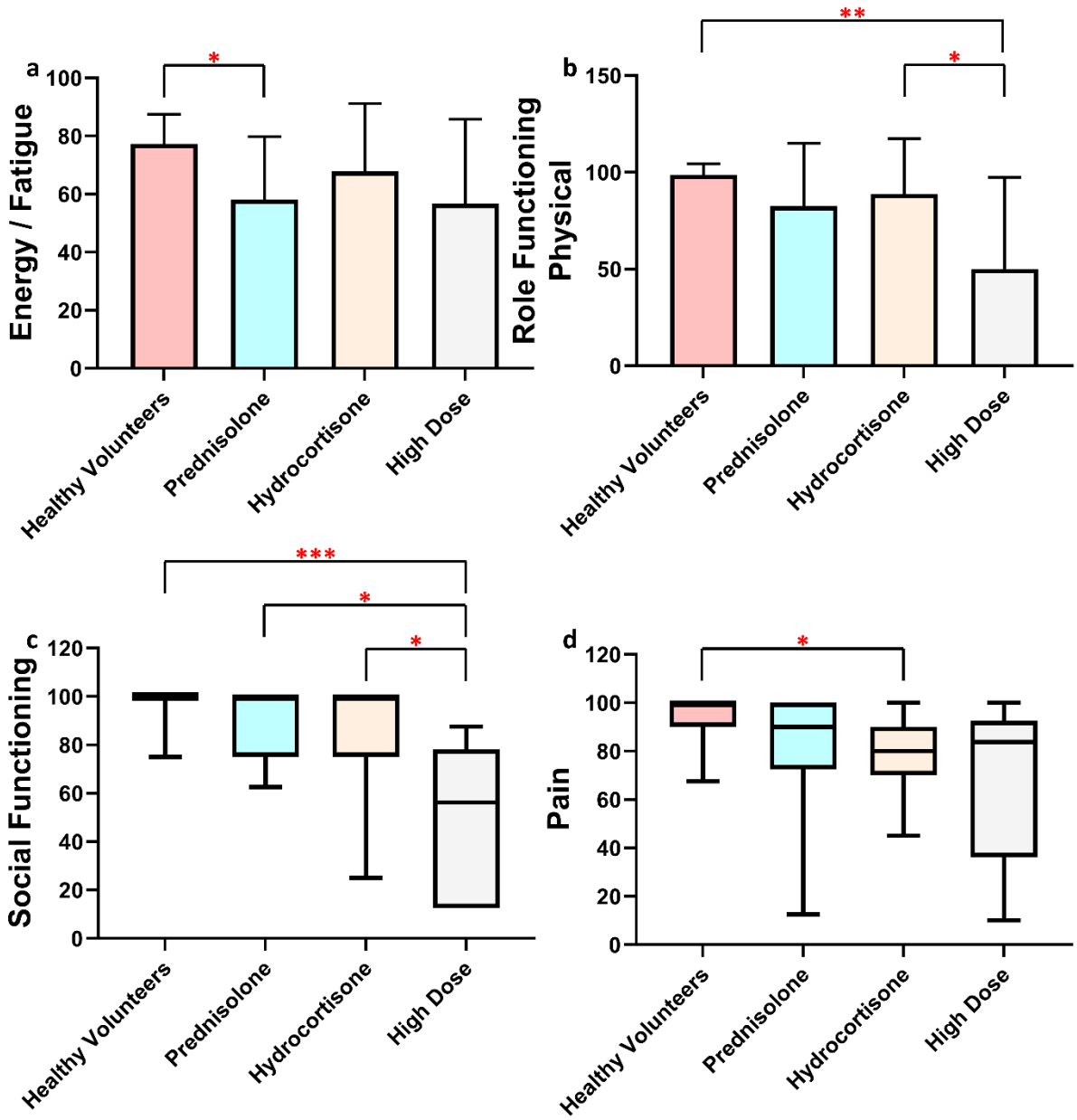

Figure 7.1- Differences between groups in Short Form Health Survey 36 (SF36) domains: Energy/Fatigue (a), Role Functioning (Physical) (b), Social Functioning (c) and Pain (d). Data is given as median, quartiles and range in (c) and (d). Data is given as mean + SD in (a) and (b). Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=20; high dose n=9. * denotes significance P<0.04 ; ** denotes significance P<0.01; *** denotes significance P<0.001

SF36 Domain	Group A Healthy Volunteers	Group B Prednisolone	Group C Hydrocortisone	Group D High Dose	Significance
Physical Functioning	100.0 (5.0)	92.5 (31.3)	92.5 (16.3)	95.0 (43.8)	P=0.90
Role Functioning (Physical)	100.0 (0)	100.0 (25.0)	100.0 (0)	50.0 (87.5)	P=0.0071*
Role Functioning (Emotional)	100.0 (0)	100.0 (0)	100.0 (0)	100.0 (75.0)	P=0.35
Energy/ Fatigue	77.3 (±10.2)	58.2 (±21.7)	67.8 (±23.5)	56.7 (±29.1)	P=0.021*
Emotional Wellbeing	88.0 (18.0)	84.0 (13.0)	84.0 (16.0)	66.0 (49.0)	P=0.098
Social Functioning	100.0 (0)	100.0 (25.0)	100.0 (25.0)	56.3 (50.0)	P=0.0008*
Pain	100.0 (10.0)	90.0 (17.5)	80.0 (15.0)	83.8 (35.6)	P=0.013*
General Health	79.3 (±15.0)	64.8 (±20.2)	62.0 (±27.7)	65.0 (±29.5)	P=0.081
Health Change	50.0 (0)	50.0 (25.0)	50.0 (25.0)	50.0 (0)	P=0.090

Table 7.1- Short Form Health Survey 36 (SF36) data for Groups A-D, separated into domains. Data is reported as mean (±SD) for parametric data and median (IQR) for non-parametric data. Healthy volunteers n=20; prednisolone n=20; hydrocortisone n=20; high dose n=9. * denotes significant P-values.

7.3.2 Crossover analysis

There were no significant differences between hydrocortisone and prednisolone in the paired analysis of the SF36 domains, for the participants who crossed over treatments.

7.4 Discussion

The SF36 data demonstrates significant differences between various groups in four of the domains. The impaired energy/fatigue score associated with prednisolone may be because prednisolone is administered once per day and titrated such that there is a low level at 8 hours after the dose (90). This pattern of optimisation would in theory lend itself to tiredness at the end of the day, which would

be mitigated by the afternoon dose of hydrocortisone in a thrice daily regimen. The significance of this finding is heightened by the fact that prednisolone would be expected to have better SF36 score compared to hydrocortisone, because the once daily mode of administration is more convenient.

The high dose glucocorticoid group showed significant impairment in role functioning (physical) and social functioning compared to healthy volunteers. Although there was also significant impairment compared to the one or more of the AI groups in these two domains, this is not a relevant finding as it involves the comparison of two different disease groups without common pathology. This finding can be explained by the severity of illness in the high dose group. As most of the Group D patients were thyroid eye disease being treated with IV methylprednisolone, they inherently possessed a threat to their sight. It is not surprising that the potential problems with vision, and general unwellness between infusions would impact the role functioning and social functioning scores.

Finally, the hydrocortisone group showed significant impairment in their pain score. It is difficult to offer an explanation for this finding. It is noticeable that there is lot of variance in the pain scores of Groups B to D compared to the healthy volunteers, but a reason for this is not forthcoming. Compared to the previously reviewed literature, reduced pain scores were in fact expected in the prednisolone cohort (285).

There is much heterogeneity in the reported findings of studies that have used the SF36 tool in AI populations. A study of 18 patients with SAI compared to 20 patient controls and 21 matched healthy volunteers (288). The SAI patients were involved in a three-arm, double-blind crossover study during which they received (a) hydrocortisone 10 mg and 5 mg, (b) hydrocortisone 10mg, 5mg and 5mg or (c) prednisone 5 mg. The blind was maintained using placebo tablets, and each of the three treatment periods lasted 4 weeks. Each patient was tracked using multiple subjective health surveys including the SF36. The (a) hydrocortisone 10 mg and 5 mg regimen proved to have significantly better scores

in physical function than both regimen (b) and prednisone in regimen (c). All patient groups showed lower physical function scores than the healthy volunteers. These findings are contrary to the present study and the previously reviewed literature. It also indicates that impaired SF36 scores cannot just be corrected by introducing more glucocorticoid replacement.

In summary, prednisolone is associated with reduced energy and increased fatigue, whilst high dose glucocorticoids are associated with worsened social functioning plus physical functioning in a role. Hydrocortisone is associated with increased pain scores. Although the first two associations are plausible, there is no overt reason why hydrocortisone would cause increased pain scores.

Chapter 8: General Discussion and Conclusion

8.1 Summary of Findings

This study has included the analysis of multiple biomarkers across different biological systems in order to compare individuals with AI prescribed prednisolone and hydrocortisone replacement, to healthy volunteers and individuals receiving high dose glucocorticoids.

Analysis of bone markers has shown no difference in the primary outcome of this study. Specifically, there is no difference in levels of OC or OC indices between any of the groups. The is however a negative correlation between prednisolone dose and uOC:cOC ratio, indicating reduced bone hormonal signalling with increasing prednisolone exposure. The negative correlation between cOC and hydrocortisone dose indicates suppression of bone formation, but there is no suggestion that hydrocortisone affects bone hormonal signalling. Further, compared to the healthy volunteers, hydrocortisone treatment is associated with increased circulating PTH. This is a signal of over-replacement, as systemic glucocorticoids will decrease intestinal and renal calcium absorption, provoking a “sub-clinical” secondary hyperparathyroidism. The results of the bone marker analysis also suggest the existence of drug-specific glucocorticoid effects that are different to the expected glucocorticoid class effects, specifically in the case of prednisolone and uOC concentrations.

The cardiovascular risk analysis has revealed a trend that AI patient have greater tendency to be receiving antihypertensive and lipid lowering treatment. This study may have been underpowered to detect an increased weight in the hydrocortisone group, but did detect increased fat mass for this cohort compared to healthy volunteers. Other anthropometric markers of risk were otherwise comparable between groups. Assessment of biochemical markers uncovered increased hs-CRP and triglycerides in the hydrocortisone group compared to the healthy volunteers. Of interest, potassium levels were markedly lower in the hydrocortisone and high dose glucocorticoid patients compared to

the healthy volunteers. The observation of lower potassium levels with hydrocortisone is supported by the crossover analysis, showing that patients who switch to hydrocortisone develop significantly lower serum potassium levels. This is probably caused by a direct renal mechanism and is unlikely to be due to MR activation.

Unexpectedly, hydrocortisone and prednisolone patients inexplicably had lower fructosamine levels than the other two groups. This is despite comparable, if not higher, HbA1c levels. This is discordant and may suggest that fructosamine should not be used as a marker of glycaemia in chronic corticosteroid use populations. It is likely that there is an uncharacterised effect of long-term glucocorticoid use at play. Insulin and its HOMA indices broadly indicated that hydrocortisone patients tend to be mildly, but significantly hyperinsulinaemic compared to healthy volunteers, with a trend towards increased insulin resistance. In accordance with the more recent interpretation of uOC as a bone hormone, the healthy volunteers showed a significant moderate correlation between HOMA-%β and uOC, suggesting that uOC can promote insulin secretion. Data from the prednisolone group indicated that with increasing uOC:cOC ratio, insulin resistance tends to go up significantly. Considered with the data showing reduction in uOC:cOC ratio with increasing prednisolone dose, these findings suggest that prednisolone may have uncharacterised actions that are not in keeping with glucocorticoid class effects. Other than this, the analysis of glycaemic markers also suggests that hydrocortisone produces mildly worse glycaemic profiles compared to the healthy volunteers, in view of the insulin and HOMA indices findings.

There was no obvious difference in the infection rates between the groups. All of the glucocorticoid groups were associated with a subclinical neutrophilia. A continuum appeared to form with high-dose glucocorticoids associated with the greatest neutrophilia, hydrocortisone causing a less severe picture, and prednisolone with the least severe neutrophilia. Hydrocortisone was also associated with a subclinical monocytophilia, whilst the high dose group tended towards monocyte suppression.

Analysis of eosinophils showed only that high dose glucocorticoids tend toward suppression of eosinophils.

Flow analysis was unfortunately underpowered. Despite this, high dose glucocorticoids were clearly associated with HLA-DR suppression in monocytes. Of note, the prednisolone group data tended toward the same direction as the high dose group in all monocyte analyses, whilst hydrocortisone tended towards the healthy volunteers. This was unexpected, as the data up to this point suggested that hydrocortisone, not prednisolone tended towards over-replacement. It is however more likely that these finding again represent prednisolone specific effects on monocyte suppression, especially as the high-dose group was made up of individuals receiving prednisolone and IV methylprednisolone. NK cell analysis hints towards high dose glucocorticoids causing changes in NK cell populations and the maturation process of NK cells. The findings did not show any difference between the groups in CD16 expression, but caution but be exercised given the low sample numbers for NK analysis in particular.

Data from the SF36 responses showed reduced role and social functioning in the high-dose glucocorticoid group, which can be expected. The key finding from this analysis was the reduced energy / fatigue score in the prednisolone group. This suggests that prednisolone may be worse for causing lethargy in AI patients. Hydrocortisone was associated with increased pain scores, but this is of doubtful real-world significance.

8.2 Remarks

This study set out to prove the following 2 hypotheses:

1- Hydrocortisone and low-dose prednisolone therapy are equivalent in safety and efficacy

2- Novel indicators from routinely measured patient parameters can be used to indicate replacement status at a single timepoint

With regards to the first hypothesis, this study has shown through assessment of multiple markers of most of the related end organ systems, that there ultimately is no difference between prednisolone and hydrocortisone. In all of the analyses performed, there have been so statistically significant differences in any single biomarker between Groups B and C. Further, none of the biomarkers have indicated issues with safety of the respective medication or its efficacy. It is therefore reasonable to conclude that both drugs are equally safe and efficacious.

This study has not answered the second hypothesis but has made progress towards achieving it. The OMNI-AID study was designed as a spectrum to have a healthy volunteer group and a high dose glucocorticoid group, with AI patients in-between them. It was intended to create a continuum of normal individuals on one end and grossly over-replaced individuals on the other. Accordingly, it was expected that the markers measured in this study would also form a scale, on which AI patients should have levels closer to the healthy volunteers than the high dose patients. Of the analytes assessed in this study, some analytes show some promise. Triglycerides, and to a lesser degree, PTH, appear to show this pattern although the high dose groups did not conform. Insulin, c-peptide and HOMA indices also showed this trend, but in the absence of data from high dose patients. More encouragingly, neutrophil count did show a trend across all groups.

The key contribution of this study is not however the identification of analytes, but the caveats that exist when trying to identify them. Specifically, we must consider the uncharacterised drug specific actions of prednisolone. This study prevents evidence that it has actions on uOC that are not consistent with hydrocortisone. Further, there appears to be prednisolone / IV methylprednisolone specific effect on monocytes. Further, the effect of both hydrocortisone and prednisolone on lowering

fructosamine have not previously been described and were unexpected. This demonstrates that care must be exercised when considering biomarkers. We must not blindly assume that all glucocorticoids exert only class effects.

A power calculation was not performed for the OMNI-AID study, as it was designed as a pilot. This has been detrimental to a number of outcomes, where there are obvious trends forming, but in the absence of sufficient power to detect them. The data from this study will prove useful in power calculations for further studies.

8.3 Further studies

Considerations for further studies have been covered in individual chapter discussions.

A prospective, randomised, double-blind crossover study would be better suited to examine the short-term markers that were used in this study. This need is being addressed by the ongoing PRED-AID study (289). As a prospective interventional clinical trial, it will provide more robust evidence than the present study.

As there is no overt difference between prednisolone and hydrocortisone in short term markers so far, it is safe to proceed to longer term studies with more robust outcomes. Any study would need to use glycaemic markers such as HbA1c, but would also benefit from 2 yearly quantitative CT or DEXA bone scans, and recording of MACE events and fractures. If possible, cardiac imaging with MRI or echocardiography would also provide valuable insights. With a large enough population and over a period of 10-plus years, such a study would provide a comprehensive picture of bone, diabetic and cardiovascular outcomes. Such a study would not be an easy undertaking as AI patients remain a rare population.

Whilst completing the immunological analysis of this study, it became apparent that there is a limited in-vivo understanding of human white cells in the context of glucocorticoid exposure, especially chronic glucocorticoid replacement. Very little research has been done in this area. For instance, changes in monocyte, basophil or eosinophil populations have not been characterised. Diurnal variation in cells following glucocorticoid exposure is not known. Functional assessments of different white cells, including neutrophils, have not been sufficiently completed in the literature. These gaps in understanding are detrimental to explaining the changes in immunity seen with different regimens.

Finding a single biomarker to gauge replacement regimens in a one stop manner remains elusive. It is not clear if a single biomarker is possible. The best prospect for one is still likely to be a white cell population, as described in the DREAM study. Unfortunately, the present study did not have sufficient power to confirm the DREAM study results. The findings of this study do support the idea that multiple markers could be combined to form an index. This would be especially useful as most of the markers examined in this study may have shown changes compared to healthy volunteers, but still remained within the reference range. By taking the analytes which showed a trend and the significant findings in this study, to put them through a principal component analysis, it should be possible to work an index that could be used a guide to replacement status.

8.4 Conclusion

By comparing a cohort of AI patients receiving hydrocortisone to healthy volunteers, the OMNI-AID study has revealed mild deterioration in multiple biomarkers associated with hydrocortisone replacement therapy. Only two parameters in this study detected relative over-replacement in the prednisolone cohort. The study has described findings that would suggest that prednisolone has drug-specific actions, that are not shared by other glucocorticoids and are not well characterised. Despite

this, the OMNI-AID study failed to find any specific differences between hydrocortisone and prednisolone cohorts in direct comparison for an exhaustive list of parameters. In the context that there is no overt difference between patients taking low-dose prednisolone and hydrocortisone, both are equally safe, efficacious and can be used interchangeably.

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Chapter 9: Appendix

9.1 Appendix 1- Related Published Papers

Endocrine CONNECTIONS

Prednisolone has the same cardiovascular risk profile as hydrocortisone in glucocorticoid replacement

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Abstract

Introduction: Patients who need glucocorticoid replacement in both primary and secondary adrenal insufficiency (AI) have the choice of either once-daily prednisolone or thrice-daily hydrocortisone. A recent European study found no difference between prednisolone and hydrocortisone users in several markers including glucose, weight, body mass index, systolic and diastolic blood pressure and waist circumference, although an increase in cholesterol and low-density lipoprotein (LDL) was suggested in a subgroup of these patients. The aim of this study was to expand the evidence base for the use of these agents as replacement therapy.
Methods: Data from 82 patients on hydrocortisone and 64 patients on prednisolone for AI at Imperial College Healthcare NHS Trust were analysed.
Results: There was no significant difference in total cholesterol, LDL levels or any other risk factors between hydrocortisone and prednisolone patients. Prednisolone was subjectively significantly more convenient than hydrocortisone ($P = 0.048$).
Conclusions: Prednisolone once daily is more convenient than hydrocortisone thrice daily, and there is no difference in the markers of cardiovascular risk measured. Because prednisolone mimics the circadian rhythm better than other glucocorticoids, it should be considered as an alternative to hydrocortisone for AI.

Key Words
- prednisolone
- hydrocortisone
- adrenal insufficiency
- cardiovascular risk

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Introduction

Adrenal insufficiency (AI) is caused either by primary adrenal failure or secondary impairment of the hypothalamic–pituitary–adrenal axis (1). Both result in glucocorticoid deficiency with additional impairment of mineralocorticoid production in primary adrenal failure. The mainstay of treatment is glucocorticoid replacement, with either hydrocortisone or prednisolone (2). Both work by binding to the glucocorticoid receptor (GR) for which prednisolone has the greater avidity (3).

Glucocorticoids in excess have a well-recognised side effect profile, commonly resulting in weight gain, hypertension, early onset diabetes and psychiatric symptoms. These are frequently seen in inflammatory or autoimmune conditions in which treatment with supraphysiological doses of exogenous steroid is required. The aim of glucocorticoid replacement therapy in adrenal failure is to reverse the deficiency using only physiological doses of steroids. Reproducing the diurnal cortisol profile

http://www.endocrineconnections.org
DOI: 10.1530/EC-17-0257

with oral medication is a significant challenge because normal cortisol production is pulsatile and consists of a circadian rhythm and an ultradian rhythm (3, 4, 5). Under-replacement may cause lethargy and an increased risk of Addisonian crises, whereas excessive replacement puts patients at the risk of Cushingoid symptoms and cardiovascular disease (6, 7). In an attempt to mimic circadian rhythmicity, hydrocortisone analogues have been developed, including dual release hydrocortisone (Duocort) (8) and delayed release hydrocortisone (Chronocort) (9). The use of subcutaneous pumps for hydrocortisone delivery has also been attempted with variable success (10). Hydrocortisone is currently the default choice for cortisol replacement as it is identical to the cortisol secreted by the adrenal glands. *In vitro* studies of the GR have further suggested that the synthetic steroids such as dexamethasone and prednisolone alter the normal transcription processes within target cells as a result of their greater avidity for the GR (4). In particular, GRs activated by synthetic glucocorticoids require significantly more time to dissociate from nuclear promoters, suggesting that steroid effects may be seen long after the synthetic glucocorticoid has been washed out.

However, hydrocortisone possesses a short half-life which prevents once-daily oral administration (11). Hydrocortisone must be taken thrice a day to ensure sufficient trough levels, but this comes at the cost of producing post-dose peaks that are not physiological, and cumulatively results in excess steroid exposure. Most patients taking hydrocortisone for AI are either over- or under-treated (11, 12). The risks of over-replacement have not been fully elucidated until recently, and are often overlooked due to an appropriate fear of Addisonian crises. However, evidence of harm from a minor excess of cortisol is apparent from patients who have subclinical autonomous cortisol production with an associated increase in morbidity and mortality from cardiovascular disease (13, 14).

Although prednisolone mimics the physiological cortisol profile more closely than hydrocortisone (15, 16), there is no evidence at present as to which steroid is more appropriate to treat AI. In the absence of such data, we offer patients either hydrocortisone (10+5+5 mg daily) or prednisolone (2–4 mg once daily). For convenience, some patients now choose the latter. To optimise the dose, patients are offered a hydrocortisone day curve (17) or a prednisolone level. We have set up our own prednisolone assay and aim for an eight-hour trough level of between 15 and 25 µg/L (http://www.imperialendo.com/prednisolone, accessed 28th July 2017) (15).

All patients on steroid replacement are regularly assessed for cardiovascular risk, as this is the commonest cause of premature death in this group (18). A recent European report found that most markers of cardiovascular risk were the same in patients on prednisolone and hydrocortisone, except for total cholesterol (TC) and low-density lipoprotein (LDL), where the values were higher in the prednisolone cohort (19). We have therefore collected data from Imperial College Healthcare NHS Trust, a tertiary centre to compare a more homogeneous group of patients on prednisolone or hydrocortisone replacement.

Methods

Data were collected from patients who were reviewed between December 2016 and May 2017 taking either prednisolone or hydrocortisone as glucocorticoid replacement therapy for primary or secondary AI at Imperial College Healthcare NHS Trust in London. Patients were between the ages of 18 and 80, had been taking the relevant steroid for more than one month and were not using any other glucocorticoids concurrently. Individuals taking glucocorticoids for suppression of autoimmune disease or other systemic disease were excluded, as were people with congenital adrenal hyperplasia. After applying these criteria, we obtained data from 146 patients, 82 of whom were taking hydrocortisone, with 64 on prednisolone. In order to ensure that there was no age bias, we also carried out a subanalysis of patients aged 18–65. All patients had gone through a normal puberty. Consent was obtained from each patient after full explanation of the purpose and nature of all procedures. As this was a study of normal patient care, and as no intervention was carried out for the purpose of this audit, ethics committee approval was not required.

We used data obtained from our routine clinical screening, analysing parameters including blood pressure, body mass index (BMI), waist-to-hip circumference ratio, lipid profile, glycosylated haemoglobin, random glucose, patient satisfaction, frequency of type 2 diabetes diagnoses and frequency of diagnosed hypertension.

The Shapiro–Wilk test was used to check for the data being normally distributed. Where data were normally distributed, Levene's test was employed to confirm homogeneity of variances between prednisolone and hydrocortisone groups, prior to subsequent analysis using Student's *t*-test (alpha level 0.05). The Mann–Whitney *U*-test was used to assess all other non-parametric data (alpha level 0.05). Data were collected and collated into

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Table 1 Demographics and characteristics of patients taking glucocorticoids as replacement therapy.

	Hydrocortisone	Prednisolone
Total patients	82	64
Mean age (S.D.)	57.3 (16.0)	52.2 (15.7)
Median age	58.0 (IQR-23)	53.5 (IQR-26)
Female (%)	62	53
Type 2 diabetes mellitus (%)	22	19
Hypertension (%)	22	22
Anti-hypertensives (%)	34	26
Statins (%)	34	25
Secondary AI (%)	74	83

Microsoft Excel 2016 (Microsoft, released 2015). Further statistical analysis was performed using IBM SPSS Statistics for Windows, Version 24.0 (IBM, released 2016).

Results

The baseline demographic data are shown in Table 1. The proportion of patients taking anti-hypertensives or statins was similar between the two groups, as were diagnoses of diabetes and hypertension. The proportion of patients with primary AI vs secondary AI was equivalent in both treatment groups. The mean total daily cumulative dose of hydrocortisone was 20.5 mg, while the mean dose of prednisolone was 3.7 mg taken once daily (Table 2). The mean hydrocortisone doses used in cases of primary AI and secondary AI were 22.3 mg and 19.9 mg, respectively. In the prednisolone group, the mean dose in primary AI was 3.9 mg compared to 3.6 mg in secondary AI. There was no difference in hydrocortisone or prednisolone doses between patients with primary and secondary AI. The distribution of doses of each drug is depicted in Fig. 1 (A and B). Two patients who were on hydrocortisone at first review chose to switch to prednisolone, so their data were included in both groups.

Our study has found no significant difference in any cardiovascular risk factors between patients taking either prednisolone or hydrocortisone replacement, apart from a slightly lower waist-to-hip ratio (WHR) in patients on prednisolone (Table 2). In particular, there was no difference in LDL or TC. We also noted significantly higher subjective satisfaction scores in the prednisolone cohort (Tables 2 and 3). The subgroup analysis of patients between 18 and 65 also found no difference in any of the factors in Table 2, between patients on prednisolone and hydrocortisone (Table 4).

Discussion

This retrospective observational study suggests that hydrocortisone and prednisolone are equivalent steroid replacement therapies with no evidence that one drug possesses a bigger cardiovascular risk than the other. While the satisfaction scores were higher in the prednisolone cohort, this finding should be viewed with caution due to the potential for variability in questioning and the fact that it may be influenced by the convenience of once-daily prednisolone dosing rather than superior control of symptoms. Similarly, the better WHR in patients on prednisolone is unlikely to be clinically significant given the lack of difference in the other parameters. It is possible

Table 2 Cardiovascular risk factors of patients taking glucocorticoids as replacement therapy.

	Hydrocortisone ($n=82$)	Prednisolone ($n=64$)	*P*-Value
Total daily dose (mg)	20.5 ($n=82$)	3.7 ($n=64$)	
Satisfaction rating	3.7 (1.2) ($n=82$)	4.1 (0.9) ($n=63$)	0.048*
Systolic blood pressure (mmHg)	129 (19) ($n=82$)	127 (18) ($n=64$)	0.579
Diastolic blood pressure (mmHg)	79 (11) ($n=82$)	77 (9) ($n=64$)	0.186
Waist circumference (cm)	101 (18) ($n=79$)	97 (13) ($n=61$)	0.354
Hip circumference (cm)	107 (15) ($n=80$)	105 (11) ($n=61$)	0.860
Waist-to-hip ratio	0.95 (0.09) ($n=79$)	0.92 (0.07) ($n=61$)	0.047*
Weight (kg)	79.8 (16.7) ($n=82$)	79.6 (15.4) ($n=64$)	0.884
Height (m)	1.67 (0.09) ($n=80$)	1.68 (0.12) ($n=62$)	0.438
Body mass index (kg/m^2)	28.8 (6.1) ($n=80$)	28.3 (5.3) ($n=62$)	0.890
HbA1c (mmol/mol)	42.7 (14.0) ($n=78$)	41.0 (11.4) ($n=62$)	0.389
Total cholesterol (mmol/L)	5.15 (1.35) ($n=81$)	4.77 (1.06) ($n=63$)	0.067
High density lipoprotein (mmol/L)	1.43 (0.44) ($n=81$)	1.33 (0.37) ($n=63$)	0.202
Low-density lipoprotein (mmol/L)	2.90 (1.10) ($n=78$)	2.75 (0.89) ($n=63$)	0.450
Random glucose (mmol/L)	6.4 (3.1) ($n=82$)	5.9 (3.0) ($n=63$)	0.106

Results are expressed as mean (S.D.). Diastolic blood pressure, height and total cholesterol were assessed using Student's *t*-test. All other data were compared using the Mann–Whitney *U*-test. Satisfaction ratings (1-very unhappy, 2-not happy, 3-neutral, 4-happy, 5-very happy).
**P*-Value <0.05.

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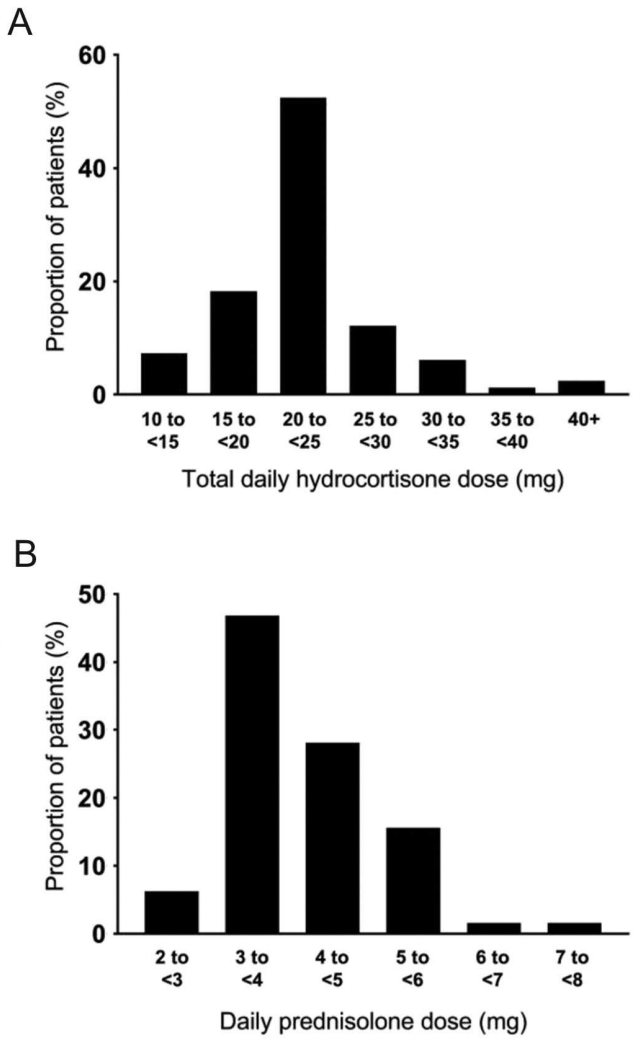

Figure 1
Frequency of the daily doses of replacement therapy taken by patients. (A) Total daily hydrocortisone dose by patients with primary and secondary AIs. (B) Daily dose of prednisolone taken by patients with primary and secondary AIs.

that patients on hydrocortisone have been on this replacement longer than those on prednisolone. As we have only been using prednisolone at our centre since 2014, long-term effects may be yet to develop.

The comparison of the two drugs in glucocorticoid replacement is a relatively unexplored area, although a cross-sectional study in 2008 showed no difference in subjective health status between 409 patients taking either prednisolone or hydrocortisone replacement (20). A more recent study also found no significant differences in the common side effects of glucocorticoids (blood pressure, HbA1c, BMI, WHR) (19), although in a subgroup of patients, higher LDL and TC levels were found in individuals taking prednisolone. It was concluded that individuals taking prednisolone therefore had a higher relative cardiovascular risk. Most patients were, however, receiving an excess of prednisolone (5–6 mg), and the data for this parameter were incomplete, being derived from only 31 patients. Furthermore, the data were collected from different centres across Europe creating exposure to confounding factors such as patient groups in one country on one drug being compared with a group elsewhere on a different drug. This was seen in a cross-sectional study comparing the two steroids, where patients in West Germany were largely treated with hydrocortisone, while those in East Germany were taking prednisolone (20). Each group will be subject to their own genotypic, phenotypic and socioeconomic influences, and consequently the results may be confounded by external factors such as genetics, wealth and standards of healthcare. We were able to minimise such variance by examining a homogeneous population who attend the pituitary or adrenal services at Imperial College Healthcare NHS Trust in London. We were also able to obtain a more complete set of data with a similar number of patients on each drug, allowing for a more objective comparison, and had values for the majority of patients in all parameters which we sought to measure. Using the same parameters as Quinkler and coworkers (19) in a more homogenous population, we have not observed the same significant differences in TC or LDL and consequent relative cardiovascular risk.

Table 3 Satisfaction ratings (1-very unhappy, 2-not happy, 3-neutral, 4-happy, 5-very happy).

	Hydrocortisone ($n=82$)	Prednisolone ($n=63$)
Happy (score =/>4) (%)	58	73
Unhappy (score =/<2) (%)	16	3

An important issue raised by our study and the one conducted by Quinkler and coworkers (19) is that of glucocorticoid dosing. The discovery of glucocorticoids in the 1940s converted conditions such as Addison's disease and many autoimmune diseases into conditions that were no longer rapidly fatal (21). Doctors have tended to over-prescribe steroids to prevent Addisonian crises without recognising the side effects of the excess (6). However, we now see an increased risk of cardiovascular death in hypoadrenal patients, probably due to excess cortisol administration, and consequently there has been a fall in the average dose of hydrocortisone prescribed. The guidelines from the endocrine society are to prescribe 3–5 mg of prednisolone daily (2), but it is likely that prednisolone is still prescribed to excess (22) as it has been found to

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Table 4 Subgroup analysis involving participants between 18 and 65.

	Hydrocortisone ($n=55$)	**Prednisolone** ($n=49$)	***P*-Value**
Mean age	48.7 (11.7)	45.9 (12.0)	
Median age	52 (IQR-21.5)	47 (IQR-17.0)	
Total daily dose (mg)	21.2 (5.9) ($n=55$)	3.7 (1.0) ($n=49$)	
Satisfaction rating	3.7 (1.3) ($n=55$)	4.0 (0.9) ($n=49$)	0.200
Systolic blood pressure (mmHg)	124 (17) ($n=55$)	122 (14) ($n=49$)	0.483
Diastolic blood pressure (mmHg)	80 (11) ($n=55$)	76 (9) ($n=49$)	0.094
Waist circumference (cm)	100 (20) ($n=54$)	95 (14) ($n=47$)	0.509
Hip circumference (cm)	108 (17) ($n=54$)	104 (10) ($n=47$)	0.638
Waist-to-hip ratio	0.93 (0.10) ($n=54$)	0.91 (0.08) ($n=47$)	0.210
Weight (kg)	80.7 (17.6) ($n=55$)	81.2 (16.3) ($n=49$)	0.656
Height (m)	1.67 (0.08) ($n=54$)	1.70 (0.12) ($n=48$)	0.119
Body mass index (kg/m^2)	29.2 (6.9) ($n=54$)	28.1 (5.8) ($n=48$)	0.608
HbA1c (mmol/mol)	43.5 (16.5) ($n=53$)	38.3 (8.3) ($n=49$)	0.211
Total cholesterol (mmol/L)	5.29 (1.36) ($n=54$)	4.88 (1.11) ($n=49$)	0.101
High density lipoprotein (mmol/L)	1.47 (0.45) ($n=54$)	1.33 (0.38) ($n=49$)	0.127
Low-density lipoprotein (mmol/L)	3.05 (1.14) ($n=52$)	2.90 (0.88) ($n=49$)	0.453
Random glucose (mmol/L)	6.3 (3.3) ($n=55$)	5.5 (2.8) ($n=49$)	0.104

Results are expressed as mean (s.d.) unless otherwise stated. Systolic blood pressure, diastolic blood pressure, height and total cholesterol were assessed using Student's *t*-test. All other data were compared using the Mann–Whitney *U*-test. Satisfaction ratings (1-very unhappy, 2-not happy, 3-neutral, 4-happy, 5-very happy).

have a potency between six and eight times higher than hydrocortisone (23). At our centre, we have been using low-dose prednisolone as our standard glucocorticoid replacement since 2014. Dosing regimens are guided using serum 8-hour prednisolone trough levels, which has resulted in our finding that low-dose replacement of 2–4 mg once daily is appropriate for most patients, and that 5 mg is excessive (http://www.imperialendo.com/prednisolone, accessed 28th July 2017) (15). The doses of glucocorticoids in this study are more equivalent to physiological requirements than those used by Quinkler and coworkers whose patients were mostly taking 5 mg daily (19).

Hydrocortisone is the native hormone cortisol, whereas prednisolone is an analogue with a double bond between positions 1 and 2 (24, 25) (Fig. 2). The use of analogues in replacement therapy is well established in modern medicine, with fludrocortisone and insulin analogues (such as insulin glargine) commonly used due to their longer half-life in comparison with native hormones. Using prednisolone in replacement therapy should have the same benefit, as the increased binding and slower dissociation (4) may reduce the risk of Addisonian crises. Hydrocortisone usage can be associated with peak levels above physiological requirements and troughs below them (11).

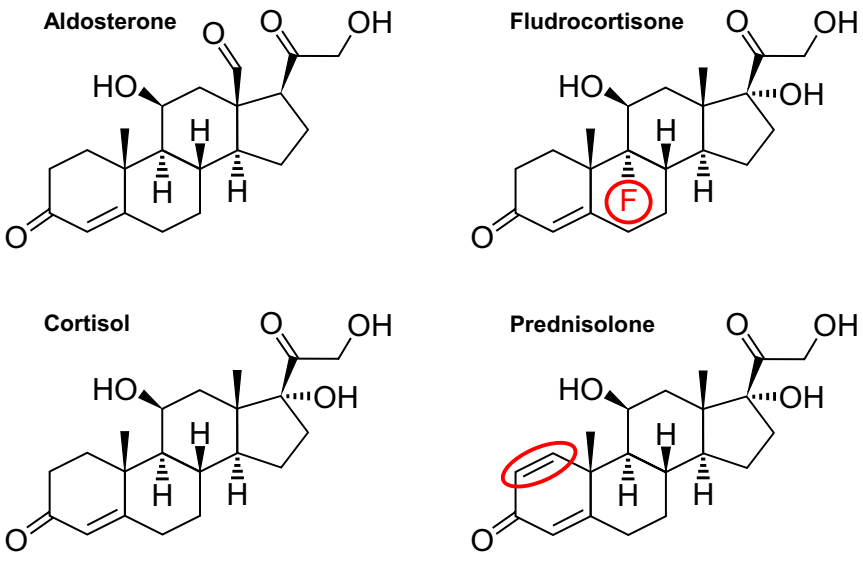

Figure 2
Biochemical structure of aldosterone, fludrocortisone, cortisol and prednisolone. The changes that give a longer half-life are shown in red. A fluorine atom is present in fludrocortisone, and a double bond in prednisolone is the only difference between these molecules and cortisol.

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In view of the longer duration of action and the ease of administration, we have been using low-dose prednisolone as our standard glucocorticoid replacement. Our findings of a similarity in side effect profiles reaffirm our preference, although these results should be interpreted with caution in view of the fact that this is a retrospective study. Blood pressure was measured at a single time point in the outpatient clinic, potentially missing the nocturnal blood pressure dip as assessed by ambulatory blood pressure monitoring. Furthermore, data were not collected to exclude participants with a familial tendency for dyslipidaemia or dysglycaemia, and analysis was not corrected for lipid lowering medication or anti-diabetic medication. A double-blind randomised controlled trial is required in order to determine whether there is any statistically significant difference in the prevalence of adverse effects of the two glucocorticoids. In the absence of evidence demonstrating superiority of one treatment above another, it is the opinion of the authors that individuals with AI should be commenced on prednisolone 3–4 mg daily, and the dose adjusted with 8-h prednisolone levels and day curves (15).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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REVIEW

The use of prednisolone versus dual-release hydrocortisone in the treatment of hypoadrenalism

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Abstract

The introduction of adrenocortical extract in 1930 improved the life expectancy of hyhpoadrenal patients, with further increases seen after the introduction of cortisone acetate from 1948. Most patients are now treated with synthetic hydrocortisone, and incremental advances have been made with optimisation of daily dosing and the introduction of multidose regimens. There remains a significant mortality gap between individuals with treated hypoadrenalism and the general population. It is unclear whether this gap is a result of glucocorticoid over-replacement, under-replacement or loss of the circadian and ultradian rhythm of cortisol secretion, with the risk of detrimental excess glucocorticoid exposure at later times in the day. The way forwards will involve replacement of the diurnal cortisol rhythm with better glucocorticoid replacement regimens. The steroid profile produced by both prednisolone and dual-release hydrocortisone (Plenadren), provide a smoother glucocorticoid profile of cortisol than standard oral multidose regimens of hydrocortisone and cortisone acetate. The individualisation of prednisolone doses and lower bioavailability of Plenadren offer reductions in total steroid exposure. Although there is emerging evidence of both treatments offering better cardiometabolic outcomes than standard glucocorticoid replacement regimens, there is a paucity of evidence involving very low dose prednisolone (2–4 mg daily) compared to the larger doses (~7.5 mg) historically used. Data from upcoming clinical studies on prednisolone will therefore be of key importance in informing future practice.

Key Words

- Adrenal
- Hypoadrenalism
- Adrenal insufficiency
- Glucocorticoid replacement
- Pituitary

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Introduction

Between 1928 and 1938, patients with Addison's disease had a 100% 5-year mortality (1). With the availability of glucocorticoid replacement therapy, initially with animal adrenocortical extract and later synthetic 11-deoxycorticosterone (2), and cortisone acetate from 1948, the prognosis of Addison's disease improved vastly. Patients were no longer dying from adrenal failure, and generous doses of glucocorticoids were given to guard against adrenal crises. Whilst the era of synthetic glucocorticoids has ushered in longer life expectancies, the use of liberal doses has come at the cost of increased long-term cardiometabolic death (3).

Half a century later, a retrospective observational study in Sweden demonstrated an increased relative risk of mortality in patients with Addison's disease compared to the general population, between 1987 and 2001 (4). The leading cause of death was cardiovascular disease, and specifically ischaemic heart disease. This was followed

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by malignancy, endocrine causes, respiratory causes and infectious diseases.

These findings were supported in a further study investigating the Swedish Addison's disease population (5). There was an overall increased standardised mortality ratio (SMR) of 2.7 for all Addison's patients compared to the general population. Again, cardiovascular disease was the commonest cause of death with malignancy coming second. Within malignancy, gastrointestinal tract cancers were the most prevalent followed by male genital cancers and non-melanoma skin cancers.

A Norwegian study demonstrated that whilst the SMR of all patients with Addison's disease was not significantly elevated compared to the general population at 1.15, there was concern for patients diagnosed under the age of 40, who had a significantly higher SMR of 1.5 (6). Cardiovascular disease, adrenal failure and cancer emerged as the top three causes. Overall, males and females diagnosed with Addison's disease could expect a life expectancy that is 3.2 and 11.2 years shorter, respectively, than their counterparts in the general population. The study did not, however, report the range of glucocorticoid doses used.

The EU-AIR registry includes secondary adrenal insufficiency and data from the UK, the Netherlands and Germany in addition to Sweden (7). In this more heterogeneous group, the main causes of death were cardiovascular disease (35%) and infection (15%). In an exclusively hypopituitary population in the USA, Mills and colleagues described a seven-fold increase in mortality when associated with adrenal insufficiency (8). Taken together, these data suggest that the mortality gap in hypoadrenalism is not just limited to primary disease.

Possible causes of the mortality gap

The cause of the aforementioned mortality gap has not been fully elucidated. Studies suggest the causes may include excess exposure to glucocorticoid replacement, under-replacement and risk of acute adrenal failure, failure to replicate the diurnal and ultradian rhythm of cortisol leading to steroid exposure at detrimental times in the day and finally, differences in the biological actions of oral synthetic glucocorticoids versus endogenous cortisol.

Interrogation of the EU-AIR registry demonstrated a higher mortality of 1.5% in patients with secondary hypoadrenalism vs 1.0% in patients with primary disease over approximately 5 years (7). In the secondary disease cohort, it was clear that those who died were in fact receiving higher doses of glucocorticoid replacement treatment, 24.0 mg of hydrocortisone vs 19.3 mg in the secondary cohort that remained alive.

These findings suggest that even very small excesses of glucocorticoid replacement may contribute towards poorer mortality outcomes. Sherlock and colleagues interrogated a regionally held UK database containing information on patients with acromegaly, which included 178 patients receiving hydrocortisone for hypoadrenalism (9). Patients with acromegaly had an increased SMR of 1.7 compared to the general population, but those on hydrocortisone showed a significant positive correlation for increasing SMR with an increasing daily hydrocortisone dose. Patients receiving greater than 30 mg of hydrocortisone daily and between 25 and 30 mg daily, had a relative risk of mortality of 2.9 and 1.6, respectively, compared to patients with acromegaly in the absence of hypoadrenalism. Even regimens greater than 20 mg of hydrocortisone may be detrimental. Evidence from Swedish populations showed that secondary hypoadrenal patients receiving such doses had a 1.88-fold increase in mortality over approximately 13 years, compared to hypopituitary patients not requiring glucocorticoid replacement. Crucially, this was not the case for those taking daily doses of 20 mg or less (10).

Escalating doses of glucocorticoid replacement are associated with worsening cardiovascular risk factors. In a three-arm crossover study, ten patients with secondary hypoadrenalism took 15 , 20 and 30 mg of hydrocortisone for 6 weeks (11). Ambulatory arterial stiffness index scores were significantly lower when participants received 15 mg of daily hydrocortisone compared to 20 and 30 mg. When secondary hypoadrenal patients were treated with 0.4–0.6 mg/kg of hydrocortisone daily, systolic and diastolic blood pressures were higher than those seen when patients received 0.2–0.3 mg/kg over a 10-week period (12). In Addison's disease, a comparison between patients on a median of 30 mg of hydrocortisone and healthy matched controls revealed increased hepatic adiposity on CT imaging, as well as a higher fasting triglycerides and lower HDL (13). These surrogate endpoints suggest that higher doses of glucocorticoid replacement induce a metabolic syndrome which likely drives an excess risk of cardiovascular disease.

Additional evidence suggests, however, that the mortality gap is not directly linked to excess glucocorticoid replacement. In a cross-sectional comparison between individuals with Addison's disease in Sweden and South Africa, it was noted that patients in Sweden received higher doses of hydrocortisone, 33.0 mg per day compared

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to 24.3 mg per day in South Africa (14). Despite being well matched and the apparent lower glucocorticoid exposure, the South African cohort had a significantly higher total cholesterol, triglycerides, and LDL, indicating a worse cardiovascular risk phenotype. Although it is possible that the observations may be due to the two sample cohorts being taken from two distinctly homogenoeus populations with their own potential genetic and environmental differences, it is important to note that the timing of doses was not considered in the study.

Loss of diurnal rhythm in autonomous cortisol secretion also increases mortality

Both autonomous cortisol secretion and oral glucocorticoid replacement therapy result in a mild excess of glucocorticoids and an altered diurnal cortisol rhythm, with supraphysiological levels particularly in the latter half of the day.

Autonomous adrenal cortisol secretion is a pathological state analogous to the proposed cause of the described mortality gap. The autonomous secretion is difficult to diagnose and detect as the excess cortisol is only slightly and not overtly raised (15). Cortisol profiles are flat, with obliteration of the physiological diurnal rhythm and morning cortisol levels may be normal. From 206 individuals followed up over 4.2 years, one study has demonstrated a 4- and 10-year reduction in life expectancy for men and women, respectively, in the UK (16). Cardiovascular disease and infectious causes were the top two causes of death. In an Italian population, patients with adrenal masses suspicious of autonomous secretion were compared to individuals with non-secreting adrenal incidentalomas (17). Those with suspicion of autonomous secretion, defined as incomplete suppression of cortisol to levels of 50–138 nmol/L after 1 mg dexamethasone suppression testing, had lower survival rates. A similarly designed Swedish study, reported greater mortality in patients with autonomous cortisol secretion (18).

A question of timing?

The normal cortisol profile has been well established and is conserved between individuals (19). Cortisol levels in humans peak at awakening, with a second peak at lunch time, and a gradual decline in levels to an overnight nadir that rises again 2–4 h before waking. Disassociation of the cortisol concentration from the expected pattern for the time of day is detrimental (20). In ten healthy individuals who were subjected to a 28-h day for 7 days, there was a 6 and 22% rise in 3-h postprandial glucose and insulin levels, compared to baseline, respectively. This observation occurred independently of fasting glucose levels, when a 12-h misalignment between the participants' circadian cycle and their behavioural cycle (or meal times) was achieved (21). Three individuals demonstrated impaired glucose tolerance in relation to meals, despite being normoglycaemic prior to the study suggesting acute insulin resistance. Mean arterial pressure was also elevated.

The disconnect between serum cortisol levels and the circadian clock maintained by all cells may be central to the adverse outcomes of shift work. It is well characterised that during shift work, there is a reversal of the diurnal cortisol rhythm, such that peak levels are seen at night, whilst individuals are awake (21, 22, 23). Charmandari *et al*. investigated peripheral blood mononuclear cells (PBMCs), sampling cells at 08:00 h and 20:00 h (24). PBMCs are easily obtainable cells that are representative of peripheral tissue. They showed a 2.8-fold greater acetylation of the glucocorticoid receptor (GR) in the morning than in the evening. With acetylation of the GR attenuating the transcriptional response of the cells to glucocorticoids, sensitivity to glucocorticoids is in inverse phase to the circadian cortisol profile. The lowest sensitivity was seen in the morning when cortisol peaks, and the highest in the evening, when cortisol wanes (25). Marked differences have already been observed in healthy individuals between glucocorticoid exposure in the morning vs the afternoon (26). Administration of 50 mg oral hydrocortisone was compared at 05:00 h and 17:00 h. The cortisol drug profiles and glucose handling parameters within the first 4 h were identical at both clock times. Between 04:00 h and 16:00 h, the peak glucose excursion, insulin secretory rates and serum insulin levels were significantly higher with the 17:00 h hydrocortisone dose as compared to the 05:00 h dose, indicating greater sensitivity to glucocorticoids later in the day.

Acetylation of the GR can be influenced by clock genes. Clock genes represent the time keeping mechanism that exists in all human cells. The intracellular equipment responsible for cellular timekeeping involves a number of feedback and transcriptional loops. Central to this is circadian locomotor output cycle kaput (CLOCK) which dimerises with brain–muscle–arnt-like protein 1(BMAL1). The CLOCK/BMAL1 dimer in turn binds to enhancer sequences in the DNA of cells to promote transcription of period genes (PER1, PER2, PER3) and cryptochromes (CRY1 and CRY2) (25). The 'master' clock in the body is

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the suprachiasmatic nucleus (SCN) in the hypothalamus, which benefits from innervation from the retina, allowing entrainment by day–night cycle (27). It is signalling from the 'master' clock which informs the 'slave' clocks residing in all other tissues that maintain synchrony (25). In the hypothalamic-pituitary-adrenal (HPA) axis, the influence of the master clock is exerted by canonical endocrine signalling via arginine vasopressin (AVP) modification of pulsatile ACTH secretion, but there also exist non-endocrine pathways with neural signalling via the splanchnic innervation of the adrenals and a local circadian clock within the adrenal glands. Apart from measurable rhythms in cortisol, GR activity is influenced by its own rhythm. CLOCK influences acetyl-transferase activity, which is directly capable of acetylating and attenuating GR function (Fig. 1) (28). This is in keeping with results from Charmandari and colleagues, where CLOCK/BMAL1 expression was relatively higher at 08:00 h, at the same time that the GR was maximally acetylated and glucocorticoid sensitivity at its lowest (24).

In addition, glucocorticoids can also affect and manipulate the timekeeping machinery of peripheral cells. Cuesta *et al.* recruited 16 healthy males, collecting PBMCs at baseline and at 6 days after the participants had taken hydrocortisone 20 mg orally every evening, 10 h post awakening (29). They found that a single dose of hydrocortisone can provoke a significant increase in PER1 mRNA expression. Further, after 6 days, PER2 levels were found to be reduced in those who responded and to have phase shifted forwards, meaning the peak levels were 9 h later than baseline. PER3 demonstrated a single pre-awakening peak at baseline, but after 6 days, a new second peak in the evening was present. Taken together, these results indicate that a single dose of hydrocortisone 20 mg in the evening can alter the expression of clock genes in peripheral cells and may in turn modify the glucocorticoid sensitivity of these cells as a result.

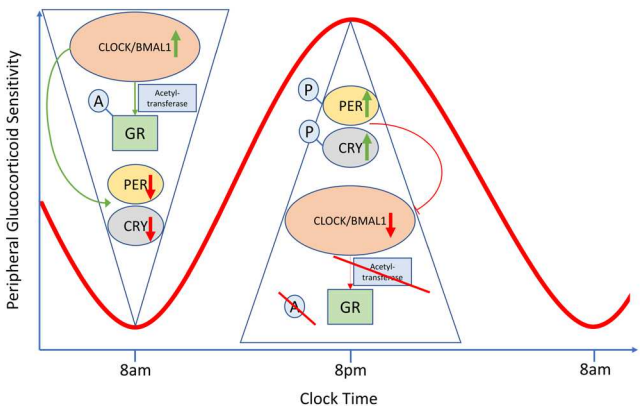

Figure 1
Model of glucocorticoid sensitivity in peripheral tissue as it fluctuates during the day (24, 25). Nadir sensitivity (peak resistance) is seen at 08:00 h, when cortisol secretion peaks. CLOCK/BMAL1 expression directly acetylates (A) the glucocorticoid receptor (GR), attenuating its function. CLOCK/BMAL1 also enhances the expression of the PER and CRY genes, although the mRNA expression is low at this time. At 20:00 h, the PER and CRY expression is high, and their phosphorylation (P) inhibits expression of CLOCK/BMAL1. The low CLOCK/BMAL1 expression, prevents acetylation of the GR, which in turn increases glucocorticoid sensitivity.

Bridging the mortality gap

The evidence presented suggests that the excess mortality seen in treated patients with adrenal insufficiency may be driven by glucocorticoid over-replacement, especially at times of increased sensitivity, such as the evening. The failure to mimic the circadian cortisol profile is central to these mechanisms. Standard-release hydrocortisone is the most common treatment used in the UK and Europe (30, 31). Its short half-life of 1.8 h mandates multiple doses per day (32, 33). The final dose exposes patients to the risk of having excess glucocorticoid in their blood at times in the day when it is potentially detrimental. As a result of its pharmacokinetic profile, oral hydrocortisone is inherently unable to mirror the circadian cortisol rhythm. The multiple dosing regimen can also result in incomplete dosing as patients may not always take hydrocortisone on time.

Continuous subcutaneous hydrocortisone infusion (CSHI) pumps, may offer a more physiological alternative cortisol replacement therapy (34), particularly for those unable to tolerate or absorb oral replacement. In an unblinded open-label feasibility study, an improvement in the vitality and physical functioning domains of the short form health survey (SF-36) measuring health related quality of life, was noted when patients were converted from oral hydrocortisone to CSHI (34). However, in a double-blind, placebo-controlled, randomised crossover trial comparing oral hydrocortisone and CSHI, there was no additional benefit seen with CSHI in subjective health scores (35). The use of CSHI requires patient training and engagement, necessitating education on pump use and maintenance. There is also a risk of local site infections and dislodgement with interruption of steroid delivery (36). The subjective health benefits of CSHI have not been conserved between studies and cardiovascular risk as assessed by anthropometric and biochemical markers, has not been adequately explored. As such, there are currently insufficient data from CSHI studies to conclude that the more physiological replacement offered translates into better long-term outcomes.

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Dual-release hydrocortisone (herein referred to as Plenadren), and prednisolone both offer a once-daily solution to glucocorticoid replacement therapy. Apart from the convenience and improved adherence to treatment with once-daily dosing, both drugs produce a smoother plasma profile (Fig. 2) (37, 38). As a result, Plenadren and prednisolone may offer better alternatives to standard-release multidose hydrocortisone, which in turn may improve the mortality outcomes.

Plenadren

Plenadren is a dual-release formulation of hydrocortisone containing both immediate release and sustained release hydrocortisone in a single tablet. Therefore it is designed to give a smoother glucocorticoid profile than standard-release hydrocortisone (39). It is available as 5 and 20 mg tablets. Data from a salivary cortisol study has shown that although morning peaks in hypoadrenal patients are equivalent to the overshoot associated with thrice daily cortisone acetate or standard hydrocortisone, Plenadren is able to generate afternoon cortisol levels that tend towards the levels seen in healthy controls (40).

In an open-label study, 64 primary hypoadrenalism patients took 3 months of thrice-daily hydrocortisone and once-daily Plenadren in a randomised crossover protocol. Patients were converted from their daily cumulative dose of thrice-daily hydrocortisone to the same total daily dose of Plenadren, administered once a day in the morning (38). Pharmacokinetic studies were performed in 18 patients. The area-under-the-curve (AUC) cortisol profile demonstrated a reduction of 19.4% in $AUC_{0–24\ h}$, for Plendaren compared to standard release hydrocortisone. There was a 6.4% increase in $AUC_{0–4\ h}$, with subsequent reductions of 30.5 and 58.8% for $AUC_{4–10\ h}$ and $AUC_{10–24\ h}$, respectively. This amounts to both, a drop in total steroid exposure and a specific reduction in the amount of steroid exposure in the afternoon and evenings. There were significant decreases with Plenadren in body weight, systolic blood pressure and diastolic blood pressure although this was tempered slightly by a significant increase in heart rate and fasting triglycerides. Further possible benefits were seen, with reductions in HbA1c by 0.1% and increase of 6.1 µg/L in procollagen type 1 N-terminal propeptide (P1NP), a bone turnover marker of osteoblastic activity. Subjective quality of life outcomes also favoured Plenadren over standard hydrocortisone. Overall, Plenadren demonstrated superiority over thrice-daily hydrocortisone in term of cardiovascular risk factors and bone health markers.

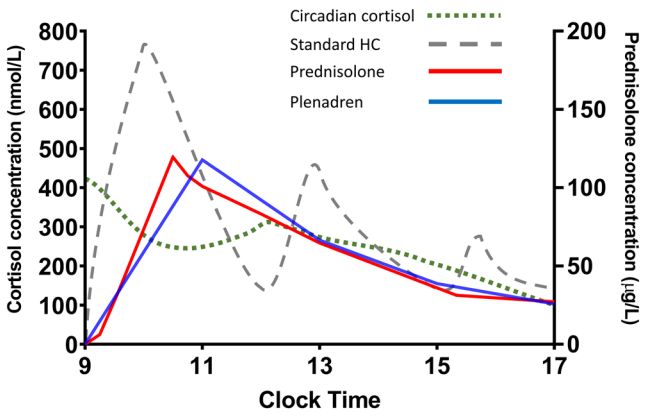

Figure 2
Serum glucocorticoid profiles of: 1-endogenous circadian cortisol (green dashed); 2-standard thrice daily hydrocortisone regimen (HC) (grey dotted); 3-prednisolone 4 mg once daily (red solid); 4-Plenadren 20 mg once daily (blue solid). (1) and (2) are plotted from data extracted from Mah *et al.* 2004 (64). (3) and (4) are generated from data collected at Imperial College Healthcare NHS Trust. Thrice daily hydrocortisone generates a peak and trough profile that both over- and under-shoot the normal cortisol profile. Plenadren and prednisolone generate a similar curve that is closer in morphology to the diurnal cortisol rhythm.

In an extension of the aforementioned study, 55 of the original 64 patients continued on Plenadren for up to 24 months after the end of the randomised crossover phase. An additional 16 patients were recruited into this study, who had not participated in the crossover trial and took Plenadren for up to 18 months (41). The safety data from this study extension showed that patients on Plenadren experienced 18.6 serious adverse events (SAEs) per 100 patient-years, compared to 13.3 SAEs per 100 patient-years on standard hydrocortisone. Notably, gastroenteritis caused hospitalisation of 11 out of the 19 patients who experienced SAEs, possibly because the dual-release formulation is more vulnerable to malabsorption in the case of intercurrent gastroenteritis. After 18 months, there continued to be a significant reduction in weight by 1.4 kg, but no change in blood pressure or HbA1c, in contrast to the 3-month data. Despite the suggestion of an increased SAE rate from the extension study, further follow-up to 5 years has confirmed that there are no significant safety concerns with the use of Plenadren and that it remains generally well tolerated (42).

In a further study, 19 individuals with Addison's disease were switched from 20 mg standard hydrocortisone in divided doses to Plenadren 20 mg once daily and evaluated over a 12-month period (43). Over 1 year, patients underwent a quarterly assessment, during which BMI, body weight and waist circumference followed an encouraging downward trend, with only

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waist circumference achieving significance. HbA1c was 2.0 mmol/mol lower at 1 year with Plenadren compared to baseline, with diabetic patients also showing improvement and lower insulin requirements. Although AddiQOL scores indicated better quality of life, the fatigue score was noted to have worsened with Plenadren. These findings are partly corroborated by a retrospective study of 49 patients with a mixture of primary and secondary hypoadrenalism, who were switched to Plenadren for a longer period of 36 months (44). Twenty-five participants were non-diabetic and 24 were prediabetic, amongst whom 30 were initially on hydrocortisone replacement and 19 on cortisone acetate. Overall, a significant reduction in BMI, waist circumference and HbA1c were observed in all patients. In addition to this, the participants with prediabetes also showed reductions in fasting insulin, insulin secretion over 2 h in response to an oral glucose tolerance test (OGTT) and an increase in both insulin sensitivity and HDL. No improvements were seen in blood pressure, and notably, 13 patients under the guidance of clinicians received a greater dose of Plenadren than expected.

There is also evidence of other metabolic benefits including improvement of hepatic steatosis (45). In 45 patients with secondary adrenal insufficiency, 25 of whom were already being treated with hydrocortisone and 20 yet to start replacement, Plenadren was administered for a 12-month period. At baseline, 31 individuals were diagnosed with steatosis on ultrasound imaging. At 12 months there were significant reductions in BMI, waist circumference, fasting insulin, insulin resistance according to homeostatic model assessment (HOMA-IR) with a corresponding increase in the insulin sensitivity. The hepatic steatosis index was noted to have significantly reduced in the cohort and the number of individuals with an index of greater than 36, came down from 33 to 11. No differences were detected in HbA1c or blood pressure, and six participants required a dose increase during the 12-month period.

The effects of Plenadren on blood pressure are difficult to interpret. Ten patients out of 17, stably replaced with cortisone acetate and diagnosed with adrenal insufficiency, were converted to Plenadren in a retrospective, case-control analysis (46). When patients were treated with Plenadren for 6 months, nocturnal diastolic pressure rose by 9 mmHg. As the relative potency of cortisone acetate and hydrocortisone is not clear, the relevance of this study to patients converting from standard release hydrocortisone to Plenadren may be limited.

A study retrospectively collected data on 14 patients who had dual energy X-ray absorptiometry (DEXA) imaging before and after switching to Plenadren (47). All patients were diagnosed with secondary hypoadrenalism, had been stable on cortisone acetate or hydrocortisone therapy for 12 months prior to the change, and had been on Plenadren for at least 2 years before the second DEXA scan. There was a significant increase in the bone mineral density in the lumbar spine and femoral neck, but not the total hip, independent of vitamin D status.

In the DREAM study, 46 patients with primary or secondary hypoadrenalism were switched from multiple daily doses of either cortisone acetate or hydrocortisone to Plenadren and were compared to 43 patients who continued on their standard regimen, as well as 25 healthy controls (48). Patients were tracked on their treatments over 24 weeks. Between the patient groups, the corrected change in body weight on Plenadren was −4.0 kg translating to a significant reduction in BMI, and waist circumference. A significant reduction was seen in HbA1c, but not fasting glucose, insulin or HOMA-IR. Hypoadrenal patients at baseline had significantly higher classical monocytes, lower non-classical monocytes and mature natural killer cells than the healthy controls. Whilst the patients on standard regimens showed no change, those who had switched to Plenadren showed normalisation of the affected immune cell populations, with their classical monocyte numbers coming down and the mature natural killer cell population rising. This coincided with a significantly better total infection scores and less flu-like illnesses in the patients on Plendaren, than those on standard regimens.

A DREAM ancillary study looked at PBMC clock gene expression in 26 of the Plenadren group, 29 of the standard treatment group and 16 of the healthy controls (49). At baseline, the hypoadrenal patients demonstrated altered expression of 19 genes including suppressed CLOCK, BMAL1 and elevated PER3 compared to the healthy controls. After 12 weeks of Plenadren, 18 of 19 genes were normalising to the levels of expression seen in the healthy volunteers. This indicates that the alterations in cellular timekeeping that are associated with traditional glucocorticoid replacement can be reversed with a more physiological glucocorticoid replacement profile.

Plenadren leads to an approximate 20% reduction in cortisol exposure in comparison to dose-matched standard hydrocortisone (38). It is distinctly possible that any notional benefits of Plenadren are solely due to this simple reduction in cortisol exposure and not necessarily due to the smoother pharmacokinetics (50).

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This exposure reduction in turn exposes patients to the risks of inadequate replacement, one manifestation of which may be higher fatigue scores recorded in subjective health scores (43). In order to mitigate against this, the current summary of product characteristics (SmPC) for Plenadren encourages individualisation of replacement doses when patients switch from other treatments (51). As a result, there is a trend towards escalation of the total daily dose in patients who have switched to Plenadren from real-world evidence (44, 45). By restoring the cortisol exposure to baseline with these dose increases, it is possible that the benefits to the surrogate endpoints seen in the randomised trials (which relied on 1:1 daily dose switching to Plenadren) may not be realised in routine clinical use.

Due to its formulation, Plenadren appears to be more vulnerable to malabsorption during intercurrent gastrointestinal disease. The sustained release preparation requires continued absorption of hydrocortisone from the gut for several hours, leading to diarrhoea as a common side effect. In addition to the caution for using Plenadren in chronic diarrhoea as noted above, there is a specific risk of hospitalisation with acute gastroenteritis (41) warranting particular mention in the SmPC as a situation where parenteral hydrocortisone may be needed (51).

Prednisolone

Prednisolone, and its prodrug prednisone were first synthesised as anti-arthritic agents in 1950 (52). They have a similar chemical structure to cortisol with an additional double bond between carbon-1 and carbon-2 (C1–C2), which increases the half-life. Oral prednisone is converted to prednisolone during first-pass hepatic metabolism by 11β-hydroxysteroid dehydrogenase type 1 and for the purposes of this review, will be considered interchangeable with prednisolone (32). The pharmacokinetic and pharmacodynamic profile of prednisolone is notably different to cortisol. It has a 2.5 and 300 times greater binding affinity to both cortisol binding globulin and albumin, respectively, when compared to cortisol (53). *In vitro* studies have demonstrated that prednisolone has a greater affinity for the GR and mineralocorticoid receptor (MR) (54). When compared to cortisol, prednisolone binds 2.26 times more avidly at the level of the GR and 1.8 times at the MR. The increased affinity, may provoke downstream genomic effects due to the increased time needed for prednisolone to dissociate from the GR compared to hydrocortisone, which in turn slows the turning off of downstream transcription on a cellular level (55). It is difficult to quantify the cumulative effects of these individual differences between prednisolone and other glucocorticoids, underlining the need for clinical studies examining the global effects on clinical outcomes such as mortality.

The C1–C2 double bond endows prednisolone with a longer half-life of up to 3.2 h, and an increased potency when compared to cortisol (32). It has long been thought that the potency of prednisolone is four times greater than hydrocortisone and this may hold true at anti-inflammatory doses (56). However, there is emerging evidence that this value understates the actual bioequivalence at lower replacement doses. A cohort of 23 individuals with a median age of 9.4 years were treated with prednisolone for congenital adrenal hyperplasia, and compared to a cohort of 21 individuals with a median age of 8.3 years who were treated with thrice-daily hydrocortisone (57). Initially the prednisolone group were prescribed 2.4–3.75 mg/m^2 of prednisolone once-daily, whilst the hydrocortisone group received 10–15 mg/m^2 in keeping with the purported bioequivalence ratio of 4:1. In order to normalise the participants' biochemical, clinical and anthropometric markers over the course of this 1-year study, the researchers were forced to reduce the amount of prednisolone prescribed on the study to 1.8–3.0 mg/m^2, whilst the patients on hydrocortisone required an increase in dose to 12–20 mg/m^2. The data from this study indicates that the potency of prednisolone may be as high as six to eight times greater than hydrocortisone, so that 3 mg of prednisolone is equivalent of 20 mg of hydrocortisone.

These findings raise questions about the true significance of studies comparing steroid replacement regimens where the ratio of 4:1 was used. In one study comparing prednisone 7.5 mg daily with hydrocortisone 30 mg daily, no difference was found between both groups in bone density (58). In a randomised, double-blind, placebo-controlled crossover study in Tunisia comparing prednisolone 5 mg and placebo versus twice-daily hydrocortisone 10 mg in fasting patients during Ramadan, there was no difference in glycaemic parameters and quality of life outcomes (59). A further study comparing 7.5 mg of prednisone with 27.8 mg of hydrocortisone found that patients on prednisone were predisposed to osteoporosis (60). It is, however, more likely that the adverse effects seen in this study were because the amount of prednisolone used was in fact equivalent to at least 45 mg of hydrocortisone, with bone turnover suppression having already been well characterised in increasing doses of hydrocortisone between 15 , 20 and 30 mg (61).

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Arriving at the minimum required dose to maintain a patient with hypoadrenalism is difficult in the absence of established biomarkers. Slowly reducing the steroid dose can be dangerous but was inadvertently undertaken in a patient with secondary hypoadrenalism, where a replacement dose of 3 mg was found to be optimal (62). There has been progress in managing hypoadrenal patients with prednisolone following the development of mass spectrometry methods to quantify prednisolone levels (37). Whilst high pressure liquid chromatography methods have been available since the 1970s, they were limited by suboptimal recovery, interference and lower limits of quantification as high as 25 µg/L (63). Mass spectrometry has allowed for an improvement in detecting prednisolone with sensitivity as low as 10 µg/L, permitting higher resolution prednisolone day curves (37). Data from six such curves in hypoadrenal patients have shown that the prednisolone profile is remarkably similar to the cortisol profile produced by Plenadren (as shown in Fig. 2). Furthermore, the data from this study demonstrates that patients can be managed on lower doses of prednisolone than previously thought, with the 3.86 mg as the average dose used, and two patients using 3 mg. It also lays the groundwork for greater individualisation of dosing regimens for patients using prednisolone, guided by 8-h serum prednisolone levels.

Data comparing 82 patients on hydrocortisone and 64 on very low-dose prednisolone has shown no difference in most anthropometric and biochemical markers of metabolic risk, such as weight, blood pressure, lipid profiles including LDL and HDL, fasting glucose or HbA1c (65). Waist-hip ratio was lower and arbitrary satisfaction with prednisolone was higher, although this may be because of the convenience of once daily administration, as opposed to thrice-daily with hydrocortisone. This study was an early demonstration that very low dose prednisolone (2–4 mg) once daily may be useful in hypoadrenalism.

Prednisolone is particularly useful in adrenal insufficiency secondary to long-term steroid use. The same principles apply in inflammatory conditions where a slow wean of prednisolone is required in order to avoid resurgence of the initial condition (66). In these circumstances, the once-daily regimen of prednisolone allows for the formulation of easy weaning protocols that are simple for patients to adhere to, and to reverse where necessary. The use of prednisolone 1 mg tablets facilitates gradual reduction in dose. Such approaches are not possible with Plenadren as the lowest denomination available is 5 mg and the dual-release formulation prohibits splitting of tablets. The same approach with standard hydrocortisone, although possible, is hindered by the complexity of modifying thrice-daily regimens and the practical difficulty and imprecision from splitting the smallest available denomination of 10 mg tablets into smaller doses.

Although both prednisolone and hydrocortisone feature on the World Health Organisation list of essential medicines, prednisolone is more widely available. Higher doses of prednisolone are used for a number of anti-inflammatory indications. As a result, patients with hypoadrenalism are routinely managed with prednisolone in many countries, but at default doses of 5 mg that are higher than the 2–4 mg which limited evidence suggests constitutes adequate replacement. Dissemination of the message that three-quarters of a 5 mg tablet is sufficient for treatment of hypoadrenalism may well have an important impact on the health of patients across the world with hypoadrenalism.

Conclusion

Emerging evidence strongly suggests that thrice-daily standard oral hydrocortisone has long-term deleterious effects. Our approach must centre on both preventing over-replacement and ensuring that there is appropriate steroid exposure at the correct times.

It may be that both Plenadren and prednisolone offer more suitable glucocorticoid replacement with concurrent cardiovascular, metabolic and immunological benefits, but there is a paucity of evidence directly comparing the two. There is also a lack of research comparing both prednisolone and Plenadren with other modalities of glucocorticoid replacement. Current studies are confounded by the relative differences in potency and dosing. Practically, both drugs offer a once-daily replacement with no current evidence of difference between the two. Further direct comparisons are therefore needed. It is hoped that ongoing trials such as PRED-AID and HYPER-AID will provide this (67, 68).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

SC is responsible for the initial draft of this manuscript. TT, KL and KM have reviewed this manuscript and made edits to the text. All authors have approved the final manuscript. All authors have contributed equally.

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9.2 OMNI-AID Study Protocol

Objective Markers and New Indicators in Adrenal Insufficiency Disease (OMNI-AID Study)

Contents **Page number (s)**

General information

Protocol title: _

Protocol version: 1.1

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Principal investigator: Prof Karim Meeran, Department of Endocrinology, Imperial College Healthcare NHS Trust.

Co-investigator: Dr Sirazum Choudhury, Department of Endocrinology, Imperial College Healthcare NHS Trust. Will recruit patients from Imperial College Healthcare NHS Trust.

Co-investigator: Prof Tricia Tan, Department of Investigative Medicine, Imperial College Healthcare NHS Trust. Will lead on trial design, submission of regulatory paperwork and trial conduct

Project summary

In Adrenal Insufficiency (AI), the body is unable to produce vital steroid hormones, the chief of these being cortisol. There are approximately 27,000 individuals in the UK with AI, with up to 3140 new cases per year. Untreated, the condition is fatal. The main treatment is replacement of the absent hormone with tablets. This is commonly done using hydrocortisone, taken 3 times daily. Correctly replacing the hormones is a significant challenge. If an individual is given too much hydrocortisone, they risk long term complications including diabetes, osteoporosis (weakened bones) and cardiovascular disease (heart attacks). If, however they are given too little, patients can feel tired, unwell and may collapse as there is insufficient steroid hormone to cope with stress.

The aim of glucocorticoid therapy is to mimic the normal cortisol day profile found in healthy individuals. The physiological diurnal cortisol rhythm involves an early morning peak in cortisol levels as individuals rise, with a gradual decline in cortisol levels through the day leading to an overnight nadir. The difficulty in mirroring this profile with oral tablets has led to many AI patients experiencing over-replacement and consequent reductions in their life expectancy.

It is not possible to use serum cortisol levels in patients with AI to accurately judge whether a patient is receiving an adequate and appropriate dose of hormone replacement therapy. In other very similar conditions such as Congenital Adrenal Hyperplasia (CAH), it is possible to use other objective markers such as growth velocity and specific precursor hormone levels to ascertain the optimum glucocorticoid dosing. Such objective markers remain elusive in AI and none have yet been described. Finding an objective marker could be key to unlocking the best clinical care and could improve mortality and morbidity in this patient group.

This pilot study will investigate novel methods to objectively ascertain whether a patient is receiving the correct dose of steroid replacement therapy. This will be done by taking a two single time-point blood tests in a selection of healthy individuals, patients with AI, patients on high dose steroids for anti-inflammatory purposes in other medical conditions, and newly diagnosed AI patients to examine whether any trends in immunological or biochemical markers can be used as indicators to gauge the adequacy of therapy.

Rationale and background information

There are approximately 27,000 individuals with Adrenal Insufficiency (AI) in the UK with an annual incidence 3140 new cases(1,2). AI is caused either by primary adrenal disease or is secondary to pituitary or hypothalamic disease. Untreated, the 2-year mortality is 85%(3). AI is treated by replacing endogenous steroids using oral glucocorticoids(4), with the goal of mimicking the physiological diurnal cortisol rhythm. Although life expectancy has improved since the introduction of treatment (5), there is still increased mortality with AI, associated with over-replacement (5,6). Over-replacement increases the long-term risks of malignancy, diabetes, cardiovascular disease and osteoporosis (5,7). If under-replaced, patients experience symptoms such as lethargy, depression, nausea and are exposed to the risk of life-threatening Addisonian crises (8). It is important to strike the correct balance with steroid replacement to optimize quality of life and prevent acute crises but without increasing the long-term risk of mortality and morbidity.

The most commonly prescribed therapies are hydrocortisone and prednisolone, accounting for the treatment of choice in 92.5% of AI patients in Europe (9). With a short half-life of 2 hours(10), hydrocortisone requires thrice-daily administration. Prednisolone is however administered once-daily.

Data on patients receiving hydrocortisone replacement therapy has indicated that up to 79% of patients are under- or over-treated when compared to the expected physiological cortisol levels expected in a healthy individual (11). Data on prednisolone replacement therapy is currently lacking. Although both prednisolone and hydrocortisone day curves may provide assistance in choosing an appropriate regimen, both procedures are time consuming and require subjective interpretation of results. Currently, there is no consistent method to objectively assess the adequacy of adrenal replacement therapy.

A recent trial comparing patients using different preparations of hydrocortisone with healthy volunteers has suggested that previously unexplored immunological cells and markers demonstrate significant differences between healthy individuals and patients with AI(12). The group have further demonstrated that the observed differences in immunomarkers and cells can be reversed towards the normal baseline as patients are treated with less total steroid exposure. This indicates that immunological cells and immunomarkers may serve as objective markers to ascertain whether a patient is being adequately treated. It is not however clear whether these markers are robust or whether they are useful to detect under-treatment as well as over-treatment.

This timely study aims to further investigate these immunomarkers in addition to other biochemical and biological markers, to better assess whether they can be used in future to judge the adequacy of steroid replacement therapy.

Study Goals and Objectives

- To measure the difference in anthropometric, biochemical, metabolic and immunological markers between healthy individuals, patients on high dose steroids for anti-inflammatory purposes in other medical conditions, patients with AI taking hydrocortisone, patients with AI taking prednisolone and newly diagnosed patients with AI. The data and samples to be collected include markers of:
    - Bone turnover
    - Cardiovascular risk
    - Glycaemic control
    - Infection rates and severity
    - Immunology profiles
    - Metabolism and glucocorticoid axis
    - Wellbeing

Study Design

This pilot study will involve 5 distinct groups:

- Group A: Healthy volunteers
- Group B: Patients on anti-inflammatory doses of steroids to treat any other medical conditions (not AI)
- Group C: Patients with treated primary or secondary AI managed with prednisolone
- Group D: Patients with treated primary or secondary AI managed with hydrocortisone
- Group E: Newly diagnosed patients with AI, on any treatment

Healthy volunteers will be recruited from the healthy volunteers' database held at the NIHR Clinical Research Facility and from the general public using adverts (newspaper and Trust intranet). Prospective healthy volunteers will be contacted from the database by using telephone calls or emails. Participants for the patient groups will be recruited to this study from inpatients and from patients attending outpatient clinics at Imperial College Healthcare NHS Trust. Patients will be identified by their direct care teams at St Mary's hospital, Hammersmith hospital and Charing Cross hospital.

Healthy volunteers will be emailed the patient information leaflet after initial contact has been made. They will be given a minimum of 24 hours to decide whether or not they wish to proceed to a screening visit, where they will be consented by a member of the research team. Participants in the patient groups will be given the patient information leaflet at clinic by the direct care team where possible. If they consent to being contacted by the research team, and were not given a patient information leaflet, it will be emailed to them by the research team at the earliest opportunity. They will be given at least 24 hours to decide whether they wish to proceed to a screening visit, during which they will be consented by a member of the research team. Completed consent forms will be stored in participants' Imperial College Healthcare NHS Trust notes.

This study will involve both healthy and patient participants attending for two visits (or three, depending on whether the screening visit and visit 1 can be combined), that are at least one week apart.

The maximum gap between study visit 1 and 2 will be 18 months. All visits will take place in the research facility. Participants will be asked to fast from 2200h the night before, taking only sips of water. In addition, they will be asked to refrain from alcohol and strenuous exercise the day before. During the first visit, the participant will be screened and if appropriate for the study, then their relevant anthropometric measurements will be recorded, bloods will be collected by a qualified member of the research team, and questionnaires completed. If it not possible to collect the relevant visit 1 study data during the screening visit (for instance if the participant did not observe an overnight fast), then the participant will be invited back on a separate occasion for visit 1. If the prospective participant is identified not to be suitable at the screening, then no blood tests will be performed and the participant will be notified of the reason why they cannot be included. Any results pertaining the screening visit will be sent to the prospective participant's GP at their request. Visit 2 will take place at least one week later and will involve collection of the same anthropometric measurements, blood samples, urine samples and questionnaires. No more than 100mls of blood will be taken per study visit (maximum 200mls total per participant in this study).

Following recruitment to this study, all participants' GPs will be notified of their participation using a standard letter. If any incidental findings are uncovered at any stage during this study, the participant will be informed by a study physician and the finding will be relayed to the participant's GP and/or direct clinical care team (where appropriate) via a letter with their consent, for further action.

If a participant loses capacity to consent to the study, they will be withdrawn from it. Participants who lose capacity or choose to withdraw, will not be invited to any further study visits, but their samples and data will continue to be stored and may be analysed as part of the study.

Healthy volunteers will not be followed up after the completion of this study. Patient participants will attend their usual outpatient clinic follow-ups at Imperial College Healthcare NHS Trust during this study, and will continue to be followed up by their direct care team as clinically appropriate after completion. There will be no study-specific follow-up. Participants will be reimbursed for expense to the amount of £20 for each completed study visit.

Primary Outcome:

- Bone health
    - assessed by measurement of change in osteocalcin, a bone formation marker

Secondary Outcomes:

- Other markers of bone health
    - assessed by measurement of change in additional bone markers and bone profile including procollagen type-1 N-terminal propeptide (P1NP), corrected calcium, parathyroid hormone (PTH), vitamin D and urinary N-terminal telopeptide (NTX).

- Surrogate markers and risk factors for cardiovascular disease
    - including anthropometric markers such as: blood pressure, heart rate, BMI, weight and waist-hip circumference ratio.
    - cardiovascular risk assessed by measurement of high-sensitivity CRP, high-sensitivity troponin I and BNP.

- Glycaemic control
    - assessed by HbA1c, fructosamine, fasting glucose levels and insulin resistance represented by HOMA-IR

- Infection rates and severity
    - assessed by completion of the German National Cohort Questionnaire (GNCQ)

- Immunology profiles
    - Assessed by measurement of soluble immunological analytes and isolated white cell populations.

- Metabolism and glucocorticoid axis
    - assessed by review of blood tests including full blood count (FBC), renal profile, liver function tests (LFTs), creatine kinase (CK), Adrenocorticotropic hormone (ACTH), cortisol, serum prednisolone (in patients taking prednisolone), cortisol binding globulin (CBG) and bicarbonate.

- Wellbeing
    - assessed by a subjective health questionnaire, the short form health survey-36 (SF-36)(13)

Inclusion Criteria:

- Aged 18 – 85 years
- Male or female
- Participants who are otherwise healthy enough to participate, as determined by pre-study medical history and physical examination

Patient groups only:

- Diagnosed with AI for over 6 months according to standard diagnostic criteria or with a medical condition requiring acute high dose steroid therapy for anti-inflammatory purposes
- If diagnosed with AI, established on stable HC replacement or prednisolone replacement, dose not altered for at least 3 months
- Established on a stable dose of Fludrocortisone, if taking, dose not altered for at least 3 months
- Participants taking other hormone replacements (e.g. levothyroxine, testosterone or growth hormone in secondary adrenal insufficiency) are accepted providing that their replacement doses have not altered for at least 3 months
- Participants who are able and willing to give written informed consent to participate in the study.

Exclusion Criteria:

- Participants with a diagnosis of Type 1 or Type 2 diabetes mellitus.

- Unable to give informed consent.
- Excessive caffeine intake above 500 mg per day.
- Taking supplements or herbal medications that the participant is unwilling or unable to stop prior to and during the study period e.g. St John's Wort (may decrease prednisolone levels), Cat's claw, Echinacea (immunomodulatory properties).
- Currently taking medications that alter CYP3A4 metabolism of glucocorticoids that the participant is unwilling or unable to stop prior to and during the study period e.g. phenytoin, phenobarbital, rifampicin, rifabutin, carbamazepine, primidone, aminogluethimide, itraconazole, ketoconazole, ciclosporin or ritonavir.
- Pregnancy, taking the oral contraceptive pill, or oral oestrogen replacement therapy due to the effects on cortisol binding globulin levels and determination of prednisolone levels. Transdermal oestrogen replacement is permitted. Females of child-bearing age will be asked to provide a urine sample for a pregnancy test at each visit.
- Diagnosis of growth hormone deficiency, untreated
- History of any medical, psychological or other condition, or use of any medications, including over-the-counter products, which, in the opinion of the investigators, would either interfere with the study.

Sample size

Up to 20 participants will be recruited to each Group (total 100 participants). Should a participant become pregnant after enrolling onto the study or decide to drop out having completed without completing the final visit, they will be withdrawn from the study and replaced with a new participant. Formal power calculations cannot be performed as this is a pilot study.

Following a pilot study to elucidate a reference range for osteocalcin in healthy volunteers and patients with AI, it has been discovered that individuals can demonstrate reductions of up to 2.4 µg/L in the short term after changes in their steroid replacement regimen. It is anticipated that that larger changes in osteocalcin will be seen when patients continue on their new regimens for a longer duration. This has been used as our primary outcome measure due to its sensitivity.

Methodology

Length of study: Typically, 2 days for each participant. This may be extended to 3 days if the visit 1 data cannot be collected on the day of screening. The minimum delay between study visits will be one week, and the maximum will be 18 months.

Aim:

To assess the differences in the following anthropometric, biochemical, immunological and overall health outcomes:

- Bone turnover
- Cardiovascular risk
- Glycaemic control
- Infection rates and severity
- Immunology profiles
- Metabolism and glucocorticoid axis
- Wellbeing

in the following groups:

- Group A: Healthy volunteers
- Group B: Patients on anti-inflammatory doses of steroids to treat any other medical conditions (not AI)
- Group C: Patients with treated primary or secondary AI managed with prednisolone
- Group D: Patients with treated primary or secondary AI managed with hydrocortisone
- Group E: Newly diagnosed patients with AI, on any treatment

to investigate whether an objective marker of adequate steroid replacement therapy can be identified.

Participant Screening

All participants will attend a screening visit prior to commencing on the study, having fasted from 2200h the night before and having taken their usual steroid replacement therapy at waking (in the relevant patient groups). At the screening visit, each volunteer will be assessed by a member of the research team, counselled and informed consent obtained. Their suitability for the study will be evaluated with a full medical history, physical examination, observations (height, weight, blood pressure, heart rate, waist circumference and hip circumference), and urinalysis.

If the volunteer is determined to be suitable, they will complete study visit 1 on the same day where possible. This may not always be possible if for instance a participant forgets to observe the overnight fast. In this case, visit 1 will be rescheduled for an alternative date.

Following recruitment to this study, all participants' GPs will be notified of their participation using a standard letter. If any incidental findings are uncovered at any stage during this study, the participant will be informed by a study physician and the finding will be relayed to the participant's GP and/or direct clinical care team (where appropriate) via a letter with their consent, for further action.

Study visits: (see Appendix 1)

There will be a 2 study visits in this trial (3, if the screening visit and study visit 1 cannot be combined). Where possible, study visit 1 will take place immediately after the screening if the patient has consented and is suitable for the study. If this is not possible (for instance, if the participant has not fasted), then study visit 1 will be arranged for another day.

Study visit 2 will take place at least one week later at the NIHR Clinical Research Facility.

For the screening and study visits, participants will be asked to attend the Research Facility at Imperial College Healthcare NHS Trust after 0800h, having fasted from 2200h the night before (permitting small sips of water only) and having taken their steroid tablets at least 2 hours prior on waking (if they are part of the relevant patient group). On arrival, observations including heart rate, blood pressure, weight and waist-hip circumferences will be recorded. The relevant blood tests, and immunological blood profiles will be performed. Urine will be requested for measurement of additional bone markers. Pregnancy tests on urine samples obtained from females of child-bearing potential will be performed at each visit. Participants will be asked to complete the SF-36 and GNCQ questionnaires during the visits. No more than 100mls of blood will be taken during a study visit. This is a total of 200mls of blood over 2 study visits by qualified members of the research team. No blood will be collected during the screening visit.

Methods of data collection and analysis

Prospective members of the patient groups will be identified by their direct care team and referred to the research team with the participant's consent.

Data will be collected and recorded during the study on both paper case record file and will be stored on NHS computers and University computers. Data stored on University Computers will be anonymised using study codes. This will include anonymised data for assessment of the primary and secondary outcomes. Electronic patient identifiable data will be transferred using only encrypted USB memory sticks and secure email (to and from NHS mail accounts, when the direct care team refers patients to the research team). All data will be transferred in an anonymised format where possible.

Patient identifiable data will be stored securely on Imperial College Healthcare NHS Trust computers.

Manual files will be stored securely in a code accessed filing room found on an access-restricted unit (the NIHR Clinical Research Facility), or in a locked filing cabinet in the Section of Investigative Medicine.

As this is a two timepoint cross-sectional study, we plan to use a paired Student t-test to compare outcome data, but will consider alternative approaches including the Wilcoxon signed-rank test. Outcome data will be compared across groups and between the two timepoints within each group. We will use a 5% level of significance for the primary endpoint and summarise all endpoints using 95% confidence intervals. We will check that the conclusions are robust for, example, to the level of the data that may be missing.

The anonymised data will be analysed will be analysed by member of the research team at Imperial College London. The results of this study will be published in peer-review journals in addition to dissemination to patient interest groups and will be made available to participants and GPs at their request.

Study operation:

The trial sponsor will be Imperial College.

Study operations will be supervised by Professor Meeran and Professor Tan with Dr Choudhury responsible for day-to-day tasks. The study will be supported by the NIHR/Wellcome Trust Clinical Research Facility.

Safety and protection of volunteers

Adverse Events – Definitions

Adverse Event (AE): any untoward medical occurrence in a patient or clinical study subject.

Serious Adverse Event (SAE): any untoward and unexpected medical occurrence or effect that:

- **Results in death**
- **Is life-threatening** – *refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe*
- **Requires hospitalisation, or prolongation of existing inpatients' hospitalisation**
- **Results in persistent or significant disability or incapacity**
- **Is a congenital anomaly or birth defect**

Medical judgement should be exercised in deciding whether an AE is serious in other situations. Important AEs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above, should also be considered serious.

Reporting Procedures

All adverse events should be reported. Depending on the nature of the event the reporting procedures below should be followed. Any questions concerning adverse event reporting should be directed to the Chief Investigator in the first instance.

Non serious AEs

All such events, whether expected or not, should be recorded.

SAEs

An SAE form should be completed and faxed to the Chief Investigator within 24 hours.

All SAEs should be reported to the London – Stanmore Research Ethics Committee where in the opinion of the Chief Investigator, the event was:

- 'related', ie resulted from the administration of any of the research procedures; and
- 'unexpected', ie an event that is not listed in the protocol as an expected occurrence

Reports of related and unexpected SAEs should be submitted within 15 days of the Chief Investigator becoming aware of the event, using the NRES SAE form for non-IMP studies. The Chief Investigator must also notify the Sponsor of all SAEs.

Local investigators should report any SAEs as required by their Local Research Ethics Committee, Sponsor and/or Research & Development Office.

Contact details for reporting SAEs

Please send SAE forms to the London – Stanmore Research Ethics Committee

Tel: 0208 104 8103

Possible Adverse Events

Throughout the study there will be at least one physician available on 24-hour call via a direct line, with a second physician on back up, and a secondary direct line to one of the senior physicians. We do not anticipate any serious adverse effects as a result of this study as there is no intervention, however participants will be provided with contact numbers and clear instructions that, if they feel unwell, they should call us.

It will be made clear to participants that they will be free to withdraw from the study at any time without providing any reason. Any possible adverse event will then be reviewed with the senior clinicians. Any serious adverse event suspected to be related to the study procedures would be reported to the ethics committee and the sponsor (Imperial College London).

Regulatory issues

Ethics approval: The Study Coordination Centre has obtained approval from the (TBC) Research Ethics Committee (REC) and Health Regulator Authority (HRA). The study must also receive confirmation of capacity and capability from each participating NHS Trust before accepting participants into the study or any research activity is carried out. The study will be conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions.

Confidentiality: The Chief Investigator will preserve the confidentiality of participants taking part in the study and is registered under the Data Protection Act.

Indemnity: Imperial College London holds negligent harm and non-negligent harm insurance policies which apply to this study.

Sponsor: Imperial College London will act as the main Sponsor for this study. Delegated responsibilities will be assigned to the NHS trusts taking part in this study.

Funding: This study does not require additional funding as the activities involved are part of routine clinical care.

Follow up

During the study, patient participants will attend their normal follow up appointments at their usual outpatient clinic in addition to the study visits. Following the completion of this study, the participants will continue life-long follow-up at their usual outpatient clinic.

Healthy volunteers will not require any follow up.

Quality assurance

The study may be subject to inspection and audit by Imperial College London under their remit as sponsor and other regulatory bodies to ensure adherence to GCP and the NHS Research Governance Framework for Health and Social Care (2nd edition).

Dissemination of results and publication policy

Results are planned to be published within scientific media but also disseminated via patient organisations such as AMEND and the Addison's Disease Self Help Group.

Consent

Consent to enter the study must be sought from each participant only after a full explanation has been given, an information leaflet offered and at least 24 hours' time allowed for consideration. Healthy volunteers will be emailed the patient information leaflet by the research team after making initial contact. A screening visit will be arranged if the participant is happy to proceed having considered the patient information leaflet. Patient participants will be given the patient information leaflet by the direct care team when they are identified as being suitable for the study at their outpatient clinic. If the patient information leaflet is not given to them, then the research team will send it via email at the earliest opportunity. Signed consent should be obtained for all participants by the research team. The right of the participant to refuse to participate without giving reasons must be respected. After the participant has entered the study the clinician remains free to give alternative treatment to that specified in the protocol at any stage if he/she feels it is in the participant's best interest, but the reasons for doing so should be recorded. In these cases the participants remain within the study for the purposes of follow-up and data analysis. All participants are free to withdraw at any time from the protocol treatment without giving reasons and without prejudicing further treatment.

Should a participant lose the capacity to consent to the study, they will be withdrawn from the study. Identifiable data or tissue already collected with consent would be retained and used in the study. No further data or tissue would be collected or any other research procedures carried out on or in relation to the participant. All participants will be informed of this at the time of consent.

Complaints

If a participant wishes to complain about their treatment, they should immediately inform the investigators (Professor Meeran, Professor Tan or Dr Choudhury). If they are not satisfied with the response, they may contact the Imperial College Joint Research Compliance Office. The normal National Health Service complaints mechanisms are also available, which includes contacting the patient advice and liaison service (PALS).

Audits:

The study may be subject to inspection and audit by Imperial College London under their remit as sponsor and other regulatory bodies to ensure adherence to GCP and the NHS Research Governance Framework for Health and Social Care (2nd edition).

Sample storage for future use:

Blood samples and urine samples obtained during this study will be stored for future analysis in ethically approved research. Participants will be informed of this at the time of providing consent. Samples will be stored in the Section of Investigative Medicine. Samples will be analysed at the Section of Investigative Medicine. Bone markers will be analysed in the Clinical Biochemistry laboratories at Imperial College Healthcare NHS Trust.

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Appendix 1: Study Visits. Participants will undergo two study visits separated by a minimum of one week. Participants in the patient groups will be asked to attend at least 2 hours after taking their usual steroid replacement therapy (whether morning or lunchtime dose). Times displayed are for example only. Where possible, the screening visit will be conducted on the same day as visit 1.

Time	**Screening Visit** *Combined with Visit 1 where possible*	**Visit 1**		**Visit 2**	
(07:00)	---	Patient Groups only: Participant takes steroid replacement tablet at home	Minimum 1- week period	Patient Groups only: Participant takes steroid replacement tablet at home	Study Complete
09:00	Informed consent Medical history Physical examination Anthropometric measurements Urinalysis (±pregnancy test)	Informed consent		Informed consent	
09:15		Obtain urine sample (NTX ±pregnancy test)		Obtain urine sample (NTX ±pregnancy test)	
09:30		Anthropometric measurements collected		Anthropometric measurements collected	
09:45		Blood test Profile 1		Blood test Profile 1	
10:00		Complete SF-36 and GNCQ		Complete SF-36 and GNCQ	

Blood profile 1:fasting renal, bone and lipid profiles, bicarbonate, full blood count (FBC), glucose, insulin, fructosamine, HbA1c, creatine kinase(**CK**), Adrenocorticotropic Hormone (ACTH), cortisol binding globulin (CBG), parathyroid hormone (PTH), vitamin D, osteocalcin (OC), procollagentype1 N-terminal propeptide(**P1NP**); hs-CRP, hs-Troponin I, BNP, soluble immunological analytes, peripheral blood mononuclear cells for white cell population analysis; **NTX:** N-terminal telopeptides; **SF-36:** Short Form health survey; **GNCQ**: German National Cohort Questionnaire;