The impact of adrenal insufficiency and its treatment on regulation of glucose, cardiovascular health, and growth parameters.

Faculty of health and life sciences

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Medicine (MD) by

Dr Julie Park

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Manuscripts currently being prepared

1. Park J, Hawcutt D, Shantsila A, Lip G, Blair J Glucose regulation and cardiovascular health in children and young people with primary adrenal insufficiency – *Intended journal: Clinical Endocrinology*
2. Park J, Hawcutt D, Shantsila A, Lip G, Blair J Glucose regulation and cardiovascular health in children and young people with secondary adrenal insufficiency – *Intended journal: Clinical Endocrinology*
3. Salivary adrenal biomarkers in healthy children - *Intended journal: Journal of clinical endocrinology and metabolism*
4. Park J, Hawcutt D, Shantsila A, Lip G, Blair J. Practicalities of assessing cardiovascular risk factors using clinic blood pressure, ambulatory blood pressure, carotid intima media thickness and flow mediated dilatation in children and young people *Intended journal: Research in Paediatrics*
5. Review of pharmacogenomics related to adrenal disease *Intended Journal: Archives of disease in childhood*

Abstract publications and international presentations

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2. P2-22 Prevalence of adrenal insufficiency (AI) requiring treatment with hydrocortisone in children tested with the LDSST Julie Park, Bhavana Selvarajah, Andrew Titman,

Joanne Blair Horm Res Paediatr 2021;94(suppl 1):1–445 DOI: 10.1159/000518849 - *presented as international poster at European Society of Paediatric Endocrinology, virtual*

3. Assessment of blood pressure and carotid intima media thickness (CIMT) in children with primary adrenal insufficiency Julie Park, Helen Shantsila, Daniel Hawcutt, Gregory Lip, Joanne Blair Horm Res Paediatr 2021;94(suppl 1):1–445 DOI: 10.1159/000518849 - *presented as international poster at European Society of Paediatric Endocrinology, virtual*
4. Outcomes of the low dose short Synacthen test in infancy. Julie Park, Lily Jones, Poonam Dharmaraj, Senthil Senniappan, Colin Morgan, Daniel Hawcutt, Joanne Blair *60th Annual Meeting of the European Society for Paediatric Endocrinology (ESPE).* Hormone Research in Paediatrics, 2022. **95(suppl 2)**(2): p. 1-616.[2] - *presented as international poster at European Society of Paediatric Endocrinology*
5. Glucose regulation and cardiovascular health in children and young people with primary adrenal insufficiency. Julie park, Daniel Hawcutt, Alena Shantsila, Gregory Lip, Joanne Blair. *60th Annual Meeting of the European Society for Paediatric Endocrinology (ESPE).* Hormone Research in Paediatrics, 2022. **95(suppl 2)**(2): p. 1-616.[2] - *presented as international poster at European Society of Paediatric Endocrinology*

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4. Salivary cortisol and cortisone in healthy children and young people Dliso Silothabo, Park Julie, Bright Orla, Jones Lily, Hawcutt Daniel, Shantsila Alena, Lip Gregory, Blair Joanne – to be presented at British Society of Paediatric endocrinology and diabetes (BSPED) November 2022

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Abbreviations

11-β HSD	11-β-hydroxysteroid dehydrogenase
11DHT	11-dihydrotestosterone
11KA4	11-ketoandrostenedione
11KT	11-ketotestosterone
11OHT	11-hydroxytestosterone
11oxC19	11-oxygenated 19-carbon
11OHA4	11-β-hydroxyandrostenedione
17OHP	17-hydroxyprogesterone
21-OH	21-hydroxylase
3-β HSD	3-β-hydroxysteroid dehydrogenase
A4	Androstenedione
ABPM	Ambulatory blood pressure monitoring
ACE	Angiotensin-converting enzyme
ACTH	Adrenocorticotropin hormone
ADH	Anti-diuretic hormone
AI	Adrenal insufficiency
AUC	Area under the Curve
AVP	Arginine vasopressin
BMI	Body mass index
BP	Blood pressure
CAH	Congenital adrenal hyperplasia
CBG	Cortico-binding globulin
CGMS	Continuous blood glucose monitoring system
CI	Confidence interval
CIMT	Carotid intima media thickness
CLIP	Corticotrophin like intermediate lobe peptide
CRF	Clinical research facility
CRH	Corticotropin releasing hormone
CT	Computerised tomography
CYP	Cytochrome P450
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DNA	Deoxyribonucleic acid
DOC	Deoxycorticosterone
DSD	Disorders of sex differentiation
FH	Familial hypercholesterolaemia
FMD	Flow mediated dilation
FSH	Follicle stimulating hormone
fT4	Free thyroxine
GCR	Glucocorticoid receptor
GH	Growth hormone
H_2O	Water
hGH	Human growth hormone
HOMA-IR	Homeostatic model assessment of insulin resistance

HPA	Hypothalamic-pituitary-adrenal axis
HPG	Hypothalamic- pituitary-gonadal axis
HSD	Hydroxysteroid dehydrogenase
IGF1	Insulin like growth factor 1
IMAGe:	Intrauterine growth restriction, metaphyseal dysplasia, adrenal hypoplasia congenita, genital abnormalities
IQR	Interquartile range
JP	Joining peptide
LC-MS	Liquid chromatography tandem mass spectrometry
LDL	Low density lipoprotein
LDSST	Low dose short Synacthen test
LH	Luteinising hormone
MC2-R	Melanocortin-2 receptors
mcg	Micrograms
mcg/m^2/day	Micrograms per metre squared per day
mg	Milligram
mg/m^2/day	Milligram per metre squared per day
mmol/L	Millimole per litre
MR-HC	Modified release hydrocortisone
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MSH	Melanocyte stimulating hormone
nmol/L	Nanomole per litre
N-POC	N-terminal pro POMC fragment
PAI	Primary adrenal insufficiency
PC1	Prohormone convertase 1
PC2	Prohormone convertase 2
PCOS	Polycystic ovarian syndrome
pmol/L	Picomole per litre
POMC	Proopiomelanocortin
PP	Precocious puberty
Pro-γ-MSH	Pro-γ-melanocyte stimulating hormone
RAAS	Renin-angiotensin-aldosterone system
SAI	Secondary adrenal insufficiency
SD	Standard deviation
SDS	Standard deviation score
SDSST	Standard dose short Synacthen test
SF-HC	Standard formulation hydrocortisone
SHBG	Sex hormone binding globulin
StAR	Steroidogenic acute regulatory
TART	Testicular adrenal rest tumour
TFT	Thyroid function test
TSH	Thyroid stimulating hormone
α-MSH	α-melanocyte stimulating hormone
β -LPH	β-lipoprotein
β-EP	β-endorphin

γ-LPH	γ-lipoprotein

Adrenal insufficiency and its impact on glucose regulation, cardiovascular health and growth parameters

Dr Julie Park

Abstract

Introduction: Cortisol affects glycaemic control, raising concerns for children with both primary (PAI) and secondary (SAI). Cardiovascular events are rare in childhood, however increased cardiovascular morbidity and mortality are described in adults. Obesity, hypertension, increased carotid intima media thickness (CIMT), decreased flow mediated dilation (FMD) and insulin resistance are independent risk factors for cardiovascular disease. Salivary hormone sampling may be useful to guide diagnosis and treatment.

Materials and methods: Two studies, one assessing children with PAI and the second assessing children with SAI, investigated glucose concentrations via continuous glucose monitoring (CGM) for seven days using Dexcom G6®. Data were compared with healthy children. Study participants also underwent assessment of clinic blood pressure (BP), ambulatory blood pressure monitoring (ABPM), measurement of CIMT and FMD, fasting blood glucose, homeostatic model assessment of insulin resistance (HOMA-IR) and lipid profiling to assess cardiovascular risk. Salivary cortisol and cortisone and other adrenal biomarker concentrations were measured throughout the day.

Results: 26 children with PAI and 20 children with SAI took part. Mean glucose concentrations were significantly higher in AI groups compared to healthy children (PAI: 6.1±0.6mmol/L); SAI:5.9±0.40mmol/L; controls 5.5±0.36mmol/L, $p<0.001$ in both cohorts). Glucose variability was not different. One child with PAI had hypoglycaemia (<3mmol/L for 2% of the time) which improved with an increase in hydrocortisone dose (9.2mg/m^2/day to 9.8mg/m^2/day). Both cohorts showed evidence of increased body mass index (BMI) and HOMA-IR, particularly the SAI group. Hypertension was prevalent in younger children with PAI on higher doses of fludrocortisone, and in SAI. Increased CIMT and reduced FMD were seen across both groups. Salivary cortisol was high in both groups, likely due to contamination with hydrocortisone. Both groups showed salivary cortisone had similar total cortisone exposure compared to healthy children. However, when pre and post dosing salivary samples were taken in the SAI group peaks and troughs were seen. Testosterone and A4 were higher in children with Addison's disease compared to the healthy population. 11KT and 11OHA4 were lower in Addison's disease. Testosterone, A4, 11KT and 11OHA4 were higher in children with congenital adrenal hyperplasia (CAH) compared to healthy children.

Discussion: Novel findings shows higher mean glucose concentrations in children with AI compared to healthy children, of which has an unclear significance. Cardiovascular risk factors are evident in childhood. Interventions to ensure a healthy lifestyle, weight management, diet and exercise are important to address regularly with children with AI. Interventions are needed to reduce cardiovascular morbidity and mortality in this cohort. Salivary hormone markers including cortisol, cortisone, testosterone, A4, 11KT and 11OHA4 may be useful for monitoring disease and treatment. 11KT and 11OHA4 needs further research to assess their underlying effect and what concentrations suggest good control of disease.

Conclusion: These two observational studies show higher mean glucose concentrations measured by CGM and increased cardiovascular risk factors in children with AI. Salivary hormones are different to those found in healthy children and may be useful markers for monitoring treatment of CAH.

1. Chapter 1: Introduction

1.1. Rationale for research

Cortisol has an important role in the regulation of growth, bone health, cognition, reproduction, immunity and inflammation, cardiovascular reactivity, electrolyte balance, glucose metabolism and, lipolysis, and plays an essential role in homeostasis [3]. Its synthesis and release from the adrenal gland in a diurnal profile is controlled by the hypothalamic-pituitary axis. Disturbance of the diurnal profile can lead to poor health outcomes including an increased risk of obesity, insulin resistance, diabetes, hypertension and premature cardiac morbidity and mortality [4].

Adrenal insufficiency (AI) may result from disorders of the adrenal gland (primary AI), or the hypothalamic-pituitary-adrenal (HPA) axis (secondary AI). All patients with AI require cortisol replacement therapy, while those with primary AI may also require aldosterone replacement therapy with the synthetic mineralocorticoid, fludrocortisone.

Cortisol deficiency in childhood is generally treated with hydrocortisone. The aim of treatment is to mimic the physiological, diurnal release of cortisol. However, treatment with standard hydrocortisone formulations results in supraphysiological cortisol concentrations following a dose of hydrocortisone, and undetectable levels prior to a dose [5, 6]. Hypoglycaemia has been reported in adult patients during periods when cortisol levels are very low, particularly overnight [7, 8]. Cortisol excess is associated with hyperglycaemia, and it is reasonable to speculate that glucose concentrations may increase following doses of hydrocortisone when cortisol concentrations are supraphysiological. To my knowledge, this has not yet been described.

Fludrocortisone is typically administered in the morning, concentrations peak after 90 minutes, and the biological half-life is 18 to 36 hours [9]. Thus, both cortisol and aldosterone profiles are non-physiological in patients with primary AI.

Morbidity and mortality are increased in adult patients with AI in part due to an excess of cardiovascular disease [10, 11]. Abnormal glucocorticoid and mineralocorticoid exposure

using hydrocortisone and fludrocortisone formulations may contribute to this increase in cardiovascular risk [12, 13].

In this thesis I will:

- Review the local practice of children with CAH compared to international guidance.
- Focus on alternative methods of monitoring cortisol, cortisone and other adrenal biomarkers by reviewing data from a cohort of healthy children, aged 5 – 18 years of age.
- Report data from the largest cohort of children and young people with primary and secondary AI to undergo CGM to date.
- Describe measures predictive of cardiovascular morbidity to determine whether interventions to improve long term health outcomes should commence in childhood.
- Describe salivary cortisol and cortisone profiling in children and young people with both primary and secondary AI.
- Report changes seen in other salivary adrenal biomarkers, namely testosterone, androstenedione (A4), 11-ketotestosterone (11KT) and 11β-hydroxyandrostenedione, in primary AI compared to the population of healthy children.
- Review the performance of low dose short Synacthen tests (LDSST) to diagnose AI in a tertiary endocrinology service.

1.2. Hypothalamic – pituitary – adrenal axis – regulation in health

The HPA axis (Figure 1-1) regulates both cortisol secretion during periods of good health and stress.

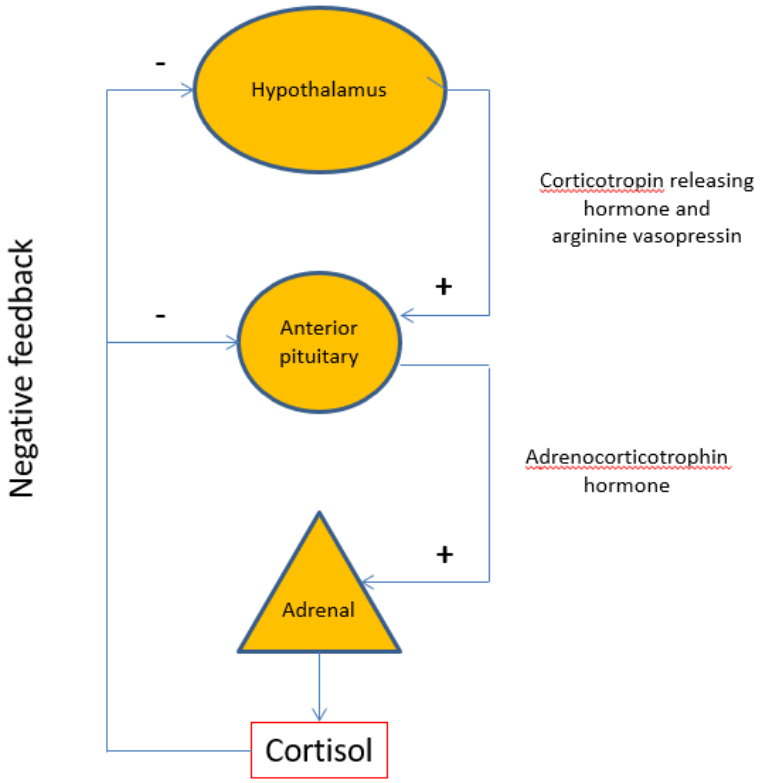


Figure 1-1:The hypothalamic-pituitary-adrenal (HPA) axis, adapted from [14]

1.2.1. Hypothalamus and Corticotropin-releasing hormone

The hypothalamus secretes corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) (Figure 1-1). CRH is released into the hypothalamo-pituitary portal system, where it acts on the anterior pituitary. AVP is transmitted to the posterior pituitary by neuronal pathways. CRH and AVP are released in response to internal stimuli from the brainstem, amygdala and hippocampus and external stimuli including stress, food and light [15]. CRH stimulates gene transcription of proopiomelanocortin (POMC). POMC undergoes extensive modification (Figure 1-2). POMC is cleaved by prohormone convertase 1 (PC1), releasing N-terminal pro POMC fragment (N-POC) and β-lipoprotein (β-LPH). N-POC is cleaved further by PC1 to form pro-γ- melanocyte stimulating hormone (Pro-γ-MSH), joining peptide (JP) and adrenocorticotropic hormone (ACTH). Prohormone convertase 2 (PC2) cleaves β- LPH into γ-

lipoprotein (γ-LPH) and β- endorphin (β-EP). PC2 can also convert ACTH into α-melanocyte stimulating hormone (α-MSH) and corticotrophin like intermediate lobe peptide (CLIP).

AVP also stimulates gene expression of POMC and therefore ACTH synthesis. Without CRH, AVP stimulates ACTH production minimally, but it has a potent synergistic role.

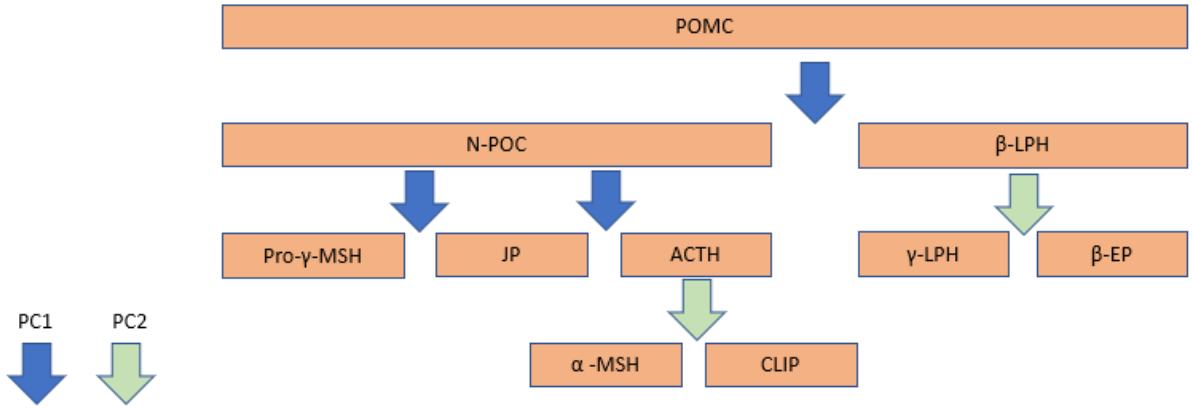


Figure 1-2: Processing and cleavage of pro-opiomelanocortin (POMC) ACTH: adrenocorticotrophic hormone, CLIP: corticotropin-like intermediate lobe protein, EP: endorphin, JP: joining peptide, LPH: lipoprotein, MSH: melanocyte stimulating hormone, N-POC: N terminal, adapted from [16]

1.2.2. Pituitary and adrenocorticotropic hormone

ACTH acts on ACTH receptors (also known as the melanocortin-2 receptors (MC2-R) in the zona fasciculata and zona reticularis of the adrenal cortex. Cholesterol is the substrate of all steroid hormones. ACTH increases the availability of cholesterol for steroid hormone biosynthesis through two pathways: (1) binding to the MC2-R, stimulating low density lipoprotein (LDL) receptor synthesis and LDL cholesterol uptake and (2) promoting the activity of steroidogenic acute regulatory (StAR) protein, which transports cholesterol from the outer to the inner mitochondrial membrane. ACTH then regulates CYP11A1 activity, initiating cleavage of the side arm of cholesterol, converting it to pregnenolone, the first step in cortisol synthesis. This process occurs in minutes and therefore a cortisol surge is seen soon after an ACTH peak.

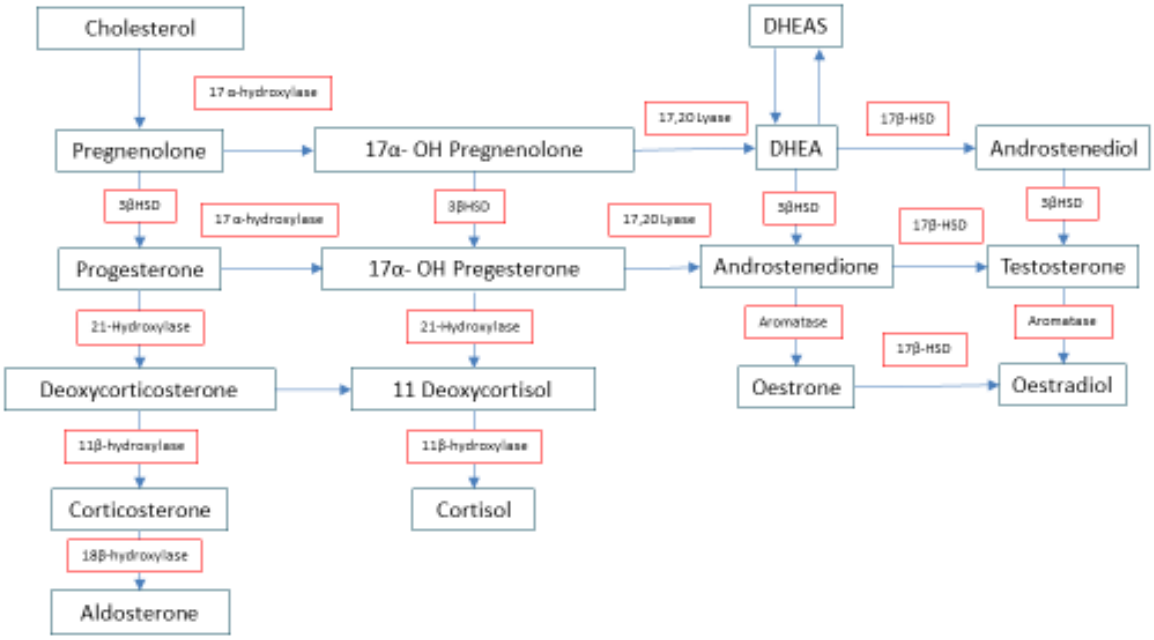


Figure 1-3: Steroidogenesis pathway. HSD: hydroxysteroid dehydrogenase, adapted from [14]

1.2.3. Adrenal gland, cortisol and cortisone

The adrenal cortex is comprised of three regions: zona glomerulosa (where cholesterol is converted to mineralocorticoids, mainly aldosterone), zona fasciculata (where cholesterol is converted to glucocorticoids, mainly cortisol) and zona reticularis (where cholesterol is converted to androgens, mainly dehydroepiandrosterone (DHEA)). Although ACTH is the main driver for cortisol synthesis and secretion, other regulatory factors include other angiotensin II, activin, inhibin, growth factors, cytokines (tissue necrosis factor (TNF-α) and leptin) and neurotransmitters [17]. Adrenal steroidogenesis is dependent on cytochrome P450 (CYP) and the hydroxysteroid dehydrogenase (HSD) enzymes [18](Figure 1-3).

Cortisol is essential to maintaining life. It increases energy sources by stimulating gluconeogenesis by enhancing expression of enzymes in the gluconeogenic pathway [19],

counter-acting the effects of insulin; inhibiting glucose uptake in adipose tissue and muscle and promoting lipolysis and protein breakdown to release substrates for gluconeogenesis [3]. Cortisol also enhances the activity of glucagon, epinephrine and other catecholamines. Glucagon further increases liver glycogenolysis, liver gluconeogenesis, liver ketogenesis, lipolysis and decreases lipogenesis [3]. Cortisol has a potent anti-inflammatory action, down-regulating pro-inflammatory proteins and up-regulating anti-inflammatory proteins.

Physiological release of cortisol follows a diurnal pattern (Figure 1-4) which is regulated by the suprachiasmatic nucleus of the hypothalamus and repeated every 24-hours. Cortisol increases from 4am, peaks thirty minutes after waking, and declines throughout the day to a nadir at 11pm [20]. This closely follows the pattern of ACTH and CRH activity [21]. The diurnal rhythm can be seen in infants as young as two months of age. Rates of cortisol secretion very widely between individuals [20, 22], and are reported to be 6.3mg/m^2/day (range 5.1-9.3) in adults and 8.0mg/m^2/day, (range 5.3-12.0) in children[20]. This wide range of cortisol exposure between individuals may represent differences in cortisol sensitivity.

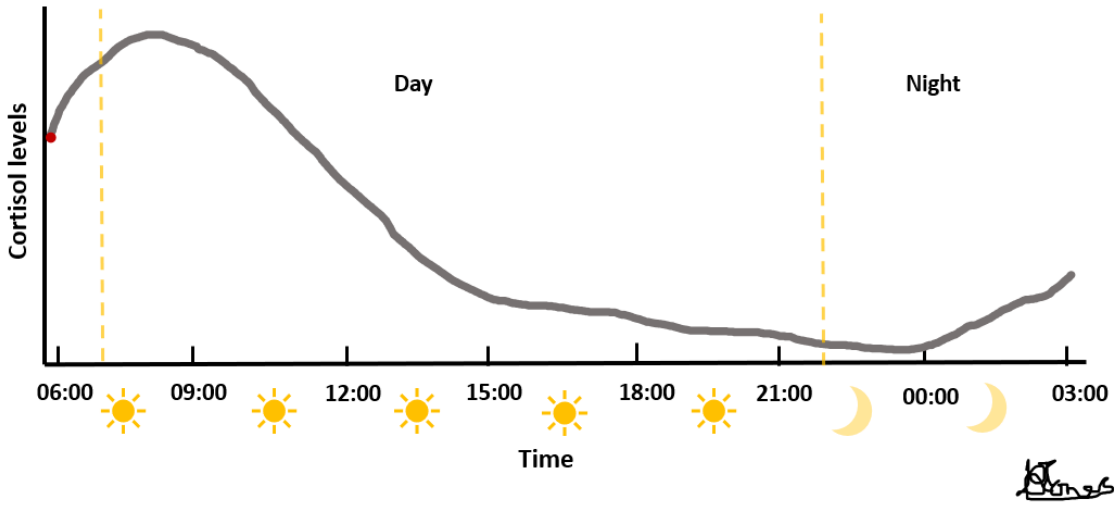


Figure 1-4: Graph showing normal diurnal rhythm, Adapted from an illustration by Lily Jones (MPhil student at the University of Liverpool), with permission

Cortisol activity is regulated by 11-β-hydroxysteroid dehydrogenase (11-β HSD). Two isoforms exist: 11-β-HSD 2 oxidises cortisol to the inactive metabolite cortisone and 11-β-HSD 1 reduces cortisone to cortisol. High concentrations of 11-β-HSD 2 are seen in the pancreas,

gastrointestinal tract, thyroid, human placenta, vascular smooth muscle cells, kidney [23] and salivary gland[24], protecting the mineralocorticoid receptor from activation by cortisol.

In plasma, 95% of cortisol is bound to proteins such as cortico-binding globulin (CBG) and albumin [25]. The remaining 5% is free and is biologically active cortisol. Active cortisol binds to glucocorticoid receptors (GCR) to assert its effects. CBG is synthesised in the liver, and synthesis increases in conditions where oestrogen levels are high (i.e., pregnancy, women taking the oral contraceptive pill) and in hyperthyroidism. Low CBG levels can be seen in hypothyroidism, hypoproteinaemia, and familial CBG deficiency [26].

1.2.4. Glucocorticoid receptors (GCR)

The GCR can be found in most cells within the body. Activation of GCR induces or suppresses target genes, which together, make up 10-20% of the human genome [27]. A single gene encodes the GCR. However, different isoforms arise from alternative splicing and translation. Differential expression of GCR isoforms determine cellular responses to glucocorticoid binding [27].

1.2.5. Altered secretory patterns within the HPA axis and its effect on health

Altered cortisol rhythms such as those seen in shift workers have been associated with poor health outcomes, including increased cardiovascular morbidity and mortality [28-31], metabolic syndrome [32] and cancer [33]. Similar cardiovascular morbidity and mortality outcomes have been seen in adults with AI who also have altered cortisol rhythms. There are no data on how early after starting shift work that these changes can be seen.

1.2.6. Response to stress

The HPA axis, alongside the sympathetic nervous system, responds to physical and emotional stressors to release energy reserves necessary to fuel the stress response. Following a stressor, the para-ventricular nucleus increases the release of CRH and AVP leading to increased secretion of ACTH and cortisol. There is rapid release and transport of cortisol to vital organs: heart, brain, muscles, resulting in an increase in BP, cardiac output and respiratory rate leading to increased oxygen and nutrient delivery. Stress increases cortisol secretion relative to the nature, intensity, and duration of stress.

1.2.7. Aldosterone and Renin -Angiotensin- Aldosterone System (RAAS)

The renin- angiotensin- aldosterone system (RAAS), shown in Figure 1-5, has a critical role in the regulation of plasma electrolytes, BP and renal blood flow. Aldosterone secretion is pulsatile and strongly related to sleep [34].

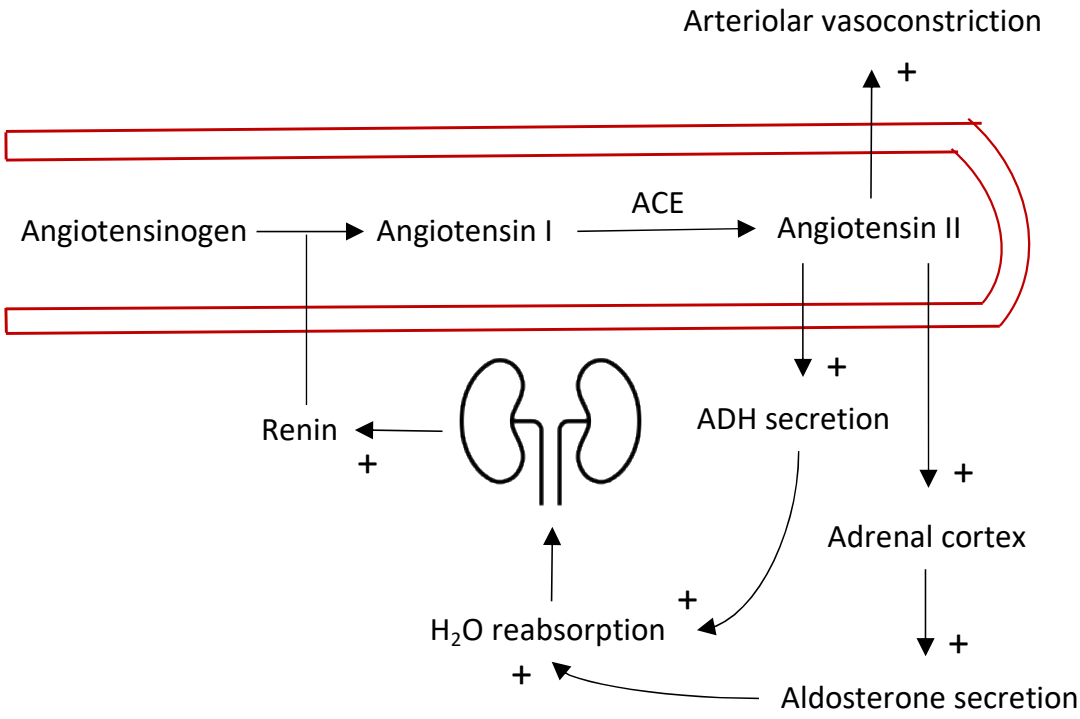


Figure 1-5: The renin-angiotensin- aldosterone pathway, illustrated by Lily Jones (MPhil student) on direct instruction of the author. ACE: Angiotensin-converting enzyme, ADH: Anti-diuretic hormone.

Aldosterone synthase, encoded by the CYP 11B2 gene, converts deoxycorticosterone (DOC) to aldosterone (Figure 1-3). Renin, secreted from the juxtaglomerular apparatus in the kidney causes cleavage of angiotensinogen to angiotensin 1, which in turn is cleaved by angiotensin-converting enzyme (ACE) into angiotensin 2, a potent stimulator of CYP 11B2 activity, promoting aldosterone activity from the zona glomerulosa. Renin is therefore elevated when aldosterone synthesis is impaired and suppressed with aldosterone excess. Aldosterone enhances sodium, potassium ATPase activity, causing sodium reabsorption in exchange for potassium excretion [35], explaining why patients with aldosterone deficiency present with hyponatraemia and hyperkalaemia.

Mineralocorticoid receptors are found in the limbic-forebrain and in tissues associated with sodium/potassium balance such as the distal renal tubule, parotid glands, colon, sweat glands and the nucleus tractus solitarii and circumventricular organs of the brain [36].

1.3. Adrenal insufficiency (AI)

1.3.1. Causes of AI

The causes of AI as defined by whether they are congenital or acquired, and caused by primary or secondary AI, are given in Table 1. The most common cause of primary AI in children is congenital adrenal hyperplasia (CAH).

Table 1.1: Table showing conditions associated with primary and secondary AI, adapted from [34]

Primary AI	
Congenital causes	
Defects of steroid biosynthesis	• CAH (21-OH deficiency, 11β-hydroxylase deficiency, 3β-hydroxysteroid dehydrogenase deficiency, 17-hydroxylase deficiency, P450 oxidoreductase deficiency), congenital adrenal lipoid hyperplasia, P450 side chain cleavage syndrome, aldosterone synthase deficiency, cortisone reductase deficiency and apparent cortisone reductase deficiency
Adrenal dysgenesis	• X-linked adrenal hypoplasia congenital, IMAGe syndrome, Pallister-Hall syndrome, pseudotrisomy 13 and Galloway-Mowat syndrome
ACTH resistance	• Familial glucocorticoid deficiency, DNA repair defect and Triple A syndrome
Cholesterol synthesis disorders	• Wolman disease, Smith-Lemli-Opitz disease, A- β-lipoproteinaemia and familial hypercholesterolaemia

Metabolic disorders: peroxisomal defects	• X-linked adrenoleukodystrophy, neonatal adrenoleukodystrophy, infantile Refsum disease and Zellweger syndrome
Acquired causes	
Autoimmune	• Isolated autoimmune adrenalitis, autoimmune polyglandular syndromes type 1, 2 and 4
Miscellaneous	• Haemorrhage, infection, trauma, surgery, infiltration and drugs (ketoconazole, rifampicin, phenytoin, phenobarbital)
Secondary AI	
Congenital causes	
	• Septo-optic dysplasia, pituitary aplasia/hypoplasia, maternal hypercortisolaemia and proprotein convertase 1 deficiency
Acquired causes	
	• Steroid withdrawal after prolonged administration, trauma, radiation therapy, surgery, tumour, infiltrative disease, and lymphocytic hypophysitis.

CAH: congenital adrenal hyperplasia, IMAGe: intrauterine growth restriction, metaphyseal dysplasia, adrenal hypoplasia congenita, genital abnormalities, DNA: deoxyribonucleic acid

1.3.2. Presentation of AI

AI may present with chronic, non-specific symptoms, including nausea, vomiting, lethargy, fatigue and abdominal pain. Alternatively, patients may present acutely with hypoglycaemia, hyponatraemia, hyperkalaemia, hypotension, shock, coma and even death.

In patients with primary AI, loss of cortisol inhibition at the level of the hypothalamus and pituitary results in high ACTH and high α-MSH concentrations (Figure 1-1) leading to pigmentation of the mucous membranes.

1.3.3. Diagnosis of AI

The diagnosis of AI is challenging. The sensitivity and specificity of diagnostic tests are debated, some tests are poorly tolerated and for all, there is a paucity of reference data from healthy infants and children.

Measurement of early morning cortisol and ACTH can be a useful screening tool and enables the distinction of primary AI from secondary or tertiary AI. The sensitivity and specificity of cortisol concentration <108nmol/L at 9am for the diagnosis of AI are reported to be 83% and 99% respectively, whilst AI is unlikely when 9am cortisol >381nmol/L [37].

Adrenal reserve can be assessed by testing the integrity of the HPA axis: insulin tolerance test; glucagon stimulation test; metyrapone test; cortico-releasing hormone test, or by directly stimulating the adrenal gland with Synacthen (synthetic ACTH). Synacthen tests are used widely in paediatric practice, as they are safer and better tolerated than other tests which have been associated with fatalities [38]. Synacthen can either be given at a 'standard dose' (250 micrograms (mcg)) (SDSST) or at lower doses (LDSST), between 300ng/m^2 body surface area to 1 mcg. The SDSST use higher doses of corticotrophin (500 times) compared to the dose that is needed to stimulate a response [39-41].The LDSST correlates well with insulin tolerance tests in adults and produces a cortisol response similar to those who have a SDSST in adults [42]. The LDSST was thought to be more sensitive for the diagnosis of AI, but less specific in a recent met-analyses of both paediatric and adult cohorts [43-46]. Inconsistencies of dosing small volumes of Synacthen required for the LDSST and adherence of the Synacthen dose to plastics have raised concerns surrounding reliability of the test [47].

Further investigations to determine the cause of AI are given in Table 1.2.

Table 1.2: Tests performed when investigating the causes of AI

Investigations	Interpretation
Primary AI	
Plasma electrolytes	Hyponatraemic and hyperkalaemia indicate mineralocorticoid deficiency
Adrenal androgens	Patients with CAH have characteristic androgen profiles. 17 hydroxyprogesterone is elevated in patients with 21-OH deficiency. Stimulation of the pathway with Synacthen may be required in mild cases.
Very long chain fatty acids	High in x-linked adrenoleukodystrophy [48]
Adrenal autoantibodies	Positive in Addison's disease [48]
Urine steroid profile	Steroid metabolites profiles are characteristic in patients with CAH due to a number of enzyme defects [49], Steroid metabolites profiles are characteristic in patients with CAH due to a number of enzyme defects [49],
Molecular genetics	Monogenic gene changes in CAH, smith-Lemli Opitz and other rare monogenic disorders A DSD gene panel may identify a diagnosis for those with abnormal genital development and AI
Imaging	
CT/ultrasound adrenals	Infiltrative disease, adrenal haemorrhage, infective disease, malignant tumours
Secondary AI	
Imaging	
Pituitary MRI	Lesions affecting pituitary or hypothalamus

CAH: congenital adrenal hyperplasia, DSD: disorder of sexual differentiation, CT: computerised tomography, MRI: magnetic resonance imaging.

1.3.4. Primary AI

1.3.4.1. CAH

CAH is the commonest cause of primary AI in childhood, and results from a reduction or complete absence of the activity of an enzyme essential for the synthesis of cortisol and aldosterone, resulting in an accumulation of hormone precursors upstream of the affected enzyme. The metabolic pathways and possible defective enzymes can be seen in Figure 1-3.

1.3.4.1.1. 21-hydroxylase deficiency

The most common cause of CAH is 21-hydroxylase (21-OH) deficiency due to mutations in the CYA21A1 gene, leading to a reduction in cortisol and aldosterone synthesis. CAH associated with 21-OH deficiency can be subclassified as classical (including salt-wasting and simple virilising) and non-classical or late onset [50].

1.3.4.1.2. Salt wasting 21-OH deficiency

Salt wasting 21-OH CAH occurs in patients that inherit two mutations that severely affect enzyme function, and usually have <1% normal 21-OH activity. Cortisol and aldosterone precursors are converted to androgens and female infants virilise in utero and present with genital abnormalities at birth. Excess androgens will not impact male genitalia, and male infants are more likely to present with adrenal crisis days to weeks following birth.

1.3.4.1.3. Simple virilising 21-OH deficiency

In simple virilising 21-OH CAH there is preservation of 1-5% of hydroxylase activity. Significant salt loss does not occur, although renin activity is often elevated. Usually, these children have two mild mutations. In this form, androgen excess will present with advanced growth and advanced skeletal maturation, and pseudo precocious puberty, which may progress to true puberty.

1.3.4.1.4. Non-classical 21-OH deficiency

Non-classical 21-OH CAH, where there is usually preservation of >5% of hydroxylase activity, is usually diagnosed in female adolescents who present with clinical features of androgen

excess, such as acne, hirsutism and a polycystic ovary syndrome (PCOS) type picture. There is usually one mild mutation. Affected girls are unlikely to require cortisol replacement.

1.3.4.1.5. Other rare forms of CAH

Cortisol synthesis is affected in other forms of CAH, including deficiencies of 3-β-hydroxysteroid dehydrogenase (3-βHSD), 11-hydroxylase and 17-hydroxylase enzymes, and by abnormalities of the StAR protein. Abnormalities of 3-βHSD and the StAR protein also lead to mineralocorticoid deficiency. Rarer causes of CAH can also present with low androgens or mineralocorticoid excess.

1.3.4.2. Addison's disease

Addison's disease is a rare, acquired auto-immune condition in which adrenal antibodies disrupt the adrenal cortex resulting in hypocortisolaemia and mineralocorticoid deficiency. It can present at any age but is more likely to present in the second or third decade of life. Diagnosis is often delayed as symptom onset is usually insidious and non-specific. Alternatively, a patient may present with adrenal crisis. Addison's disease can be isolated or occur alongside other autoimmune conditions as part of a polyglandular syndrome.

1.3.5. Secondary AI

Secondary and tertiary AI may be complicated by deficiencies of other anterior pituitary hormones. Involvement of the posterior pituitary results in diabetes insipidus (DI). These hormone pathways will also require hormone replacement, which may not accurately mimic physiological secretion. Mineralocorticoid pathways are less likely to be affected as the renin-angiotensin-aldosterone pathway maintains mineralocorticoid homeostasis. ACTH has no effect on aldosterone synthase (CYP11B2) gene or enzyme activity [35], thus the mineralocorticoid pathway remains intact.

Congenital secondary AI may be associated with learning difficulties, visual disturbance and complex neuro-disability. Hypothalamic involvement may result in hypothalamic syndrome.

1.3.6. Treatment of AI

1.3.6.1. Pharmacokinetics of hydrocortisone

All forms of AI are treated by replacing cortisol with exogenous steroids. Hydrocortisone is used in preference to synthetic glucocorticoids because it is of lower potency, may be associated with fewer side effects and can be titrated in smaller increments [51]. Cortisol concentrations peak thirty minutes after administration and hydrocortisone has a half-life of ninety minutes. Therefore, multiple doses are required throughout the day to maintain serum cortisol concentrations, resulting in supra-physiological peaks thirty minutes after each dose, followed by troughs prior to the next dose (Figure 1-6).

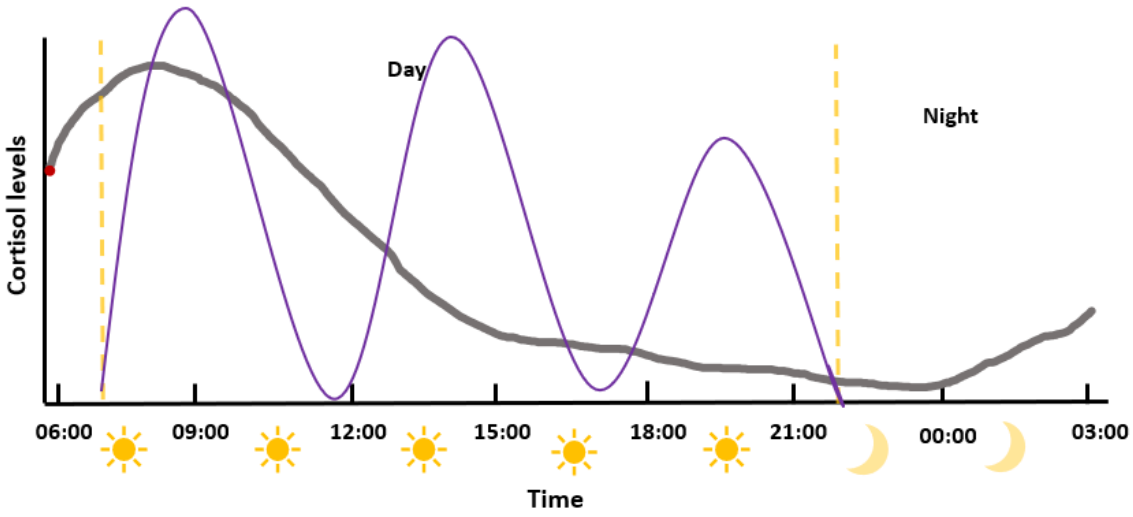


Figure 1-6: graph showing cortisol concentrations following standard formulation hydrocortisone (purple line) on the background of a normal diurnal rhythm (black line), Adapted from illustration by Lily Jones (MPhil student at the University of Liverpool)

Following a dose of hydrocortisone, cortisol concentrations may exceed the binding capacity of CBG and other binding proteins, resulting in a rapid increase in free cortisol and urinary clearance [51]. For this reason, increases in hydrocortisone doses leads to an increase in peak hydrocortisone exposure to a greater degree than total cortisol exposure [52], and the 'area under the curve' (AUC) of cortisol concentrations are not directly proportional to dose size [53].

Marked inter- and intra-individual variability in cortisol profiles are reported, even following intravenous doses [5, 52], with the AUC varying tenfold between individuals on oral hydrocortisone.

In patients with CAH, supraphysiologic doses of glucocorticoids are needed to suppress ACTH and reduce drive by the adrenal gland to secrete androgens. It can be a difficult balance to avoid glucocorticoid deficiency risking adrenal crisis, and iatrogenic glucocorticoid excess.

1.3.6.2. Hydrocortisone Formulations

Hydrocortisone can be given as an immediate release tablet, a liquid solution and granules (Alkindi®) as standard formulations (SF-HC). Newer, modified release hydrocortisone (MR-HC) have been introduced, aiming to mimic a more physiological glucocorticoid profile. These include Plenadren® and Efmody®, previously known as Chronocort®. Plenadren® has a hydrocortisone coating that is released immediately and has an extended-release core, licensed for once-daily dosing in adult patients with AI [51]. This gives an extended profile of serum cortisol compared to SF-HC. In adults a single dose of Plenadren® gives a similar cortisol profile to thrice daily hydrocortisone, but with higher cortisol levels in the morning and lower in the evening [54](Figure 1-7). Studies in patients treated with Plenadren reported improvements in metabolic profiles, weight and BP [55] and quality of life [56, 57]. This formulation is unlikely to control the early morning rise in androgens in patients with CAH as the early morning rise in cortisol is not replicated.

Cortisol concentrations following a dose of Efmody® show lower concentrations shortly after a dose with a late rise in cortisol concentration towards the end of the dose, [58](Figure 1-8). Efmody® is used as a twice daily regimen with the intention of replicating the pre-waking rise in cortisol levels, thus reducing ACTH drive to the adrenal glands [59]. Superior control of androgens is reported during treatment with Efmody® compared to thrice daily SF-HC [60, 61]. Hydrocortisone pumps, aiming to mimic diurnal cortisol profiles have also been trialled.

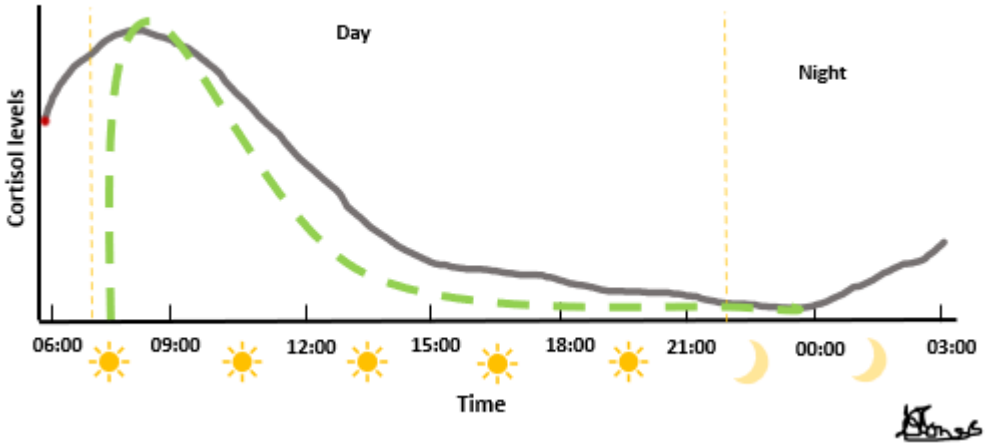


Figure 1-7: Graph showing release of Plenadren (green, dashed line) compared to normal diurnal rhythm (black, solid line) - adapted from illustration by Lily Jones (MPhil student at the University of Liverpool), with permission

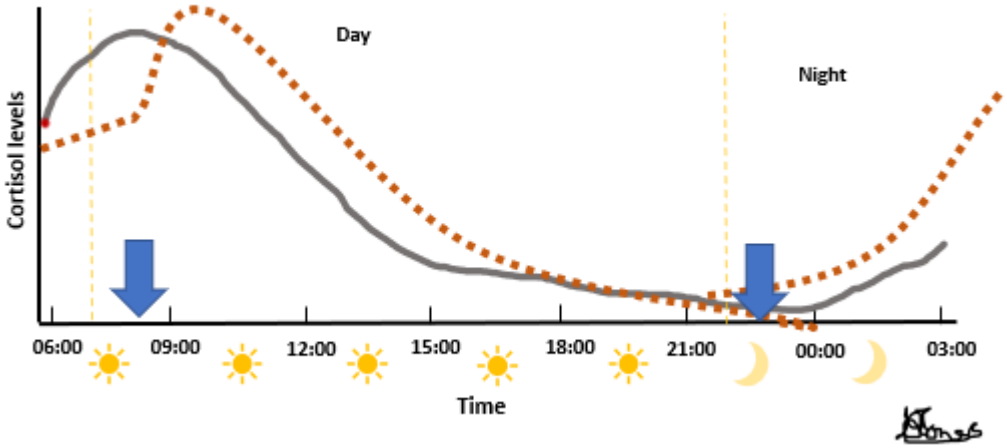


Figure 1-8: Graph showing release of twice daily Efmody (brown dotted line) compared to the diurnal rhythm of cortisol (black line), redrawn from Diurnal website using illustration by Lily Jones, MPhil student. (Blue arrows indicate when Efmody dose is administered)

1.3.6.3. Mineralocorticoid formulations

Treatment of primary AI may also require aldosterone replacement with fludrocortisone. Physiological aldosterone secretion is pulsatile and strongly related to sleep [34]. Fludrocortisone is typically administered in the morning, concentrations peak after 90 minutes, and the biological half-life is between 18 to 36 hours [9]. Infants with CAH are often resistant to aldosterone in the first few years of life [75-77], when high doses of

fludrocortisone, together with salt supplementation, are required. Both cortisol and aldosterone profiles are non-physiological in patients with primary AI.

1.3.7. Monitoring patients with AI

1.3.7.1. Clinical monitoring in all patients with AI

Patients with AI require regular monitoring for the effects of under and over treatment, including assessments of growth, body weight, BP, mood and fatigue [48]. Excessive weight gain with reduced height velocity or other symptoms or signs of Cushing's disease suggest over treatment. Inadequate weight gain, fatigue, anorexia and hyperpigmentation suggest undertreatment of glucocorticoids. In patients with CAH, under treatment of glucocorticoids can also be associated with excess androgens leading to hirsutism, amenorrhoea, abnormal or early puberty and infertility.

In those requiring mineralocorticoid replacement, signs and symptoms of inadequate replacement include poor weight gain, salt craving and dehydration. Excessive replacement can result in hypertension. BP monitoring should be performed routinely.

It is recommended that infants are assessed more regularly, every three to four months to assess growth, BP and general well-being [48]. Sensitivity to mineralocorticoid increases over the first few months of life, therefore BP is particularly important to measure.

Patient education regarding increasing the dose of glucocorticoids during illness is important, as is educating the child and their family regarding signs and symptoms of an impending adrenal crisis.

1.3.7.2. Biochemical monitoring in AI

Biochemical monitoring should include electrolytes to assess for hyponatraemia and hyperkalaemia. If a patient is symptomatic of hypo or hypercortisolaemia, a cortisol day curve may be considered.

1.3.7.2.1. Biochemical monitoring particular to primary AI

In primary AI, renin and aldosterone are used to assess adequacy of fludrocortisone dosing. A high normal, or slightly raised plasma renin activity is recommended [48]. In CAH, blood spot monitoring of 17hydroxyprogesterone (17OHP) levels in 21-OH CAH can guide treatment. Testosterone, A4, sex hormone binding globulin (SHBG) and dehydroepiandrosterone sulphate (DHEAS) monitoring will also assess androgen excess. If

there are signs of early or delayed puberty, gonadotrophins may be measured. In children and young people with autoimmune primary AI screening for other autoimmune disease can be considered periodically [48].

1.3.7.2.2. Biochemical monitoring relevant particular to secondary AI

In those with secondary AI, other pituitary hormones should also be monitored and treated adequately to reduce disease burden.

1.4. Glucose profiles in AI

1.4.1. Importance of cortisol in glucose regulation

It has long been known that adrenal disease can be associated with disordered glucose control [62]. Patients with undiagnosed AI are susceptible to hypoglycaemia [62, 63], particularly during the paediatric period [64], while cortisol excess may be associated with hyperglycaemia and insulin resistance.

1.4.2. Hypoglycaemia in patients with AI

Patients treated with hydrocortisone may be vulnerable to periods of hypoglycaemia when cortisol concentrations fall between hydrocortisone doses, particularly during the overnight fasting period. Children, who take the final dose of hydrocortisone earlier than adults and have a longer overnight fast, may be particularly susceptible to overnight hypoglycaemia. To my knowledge, only two paediatric studies have reported prospective glucose profiles in children with AI [65, 66]. One other study reviewed causes of adrenal crisis and hypoglycaemia retrospectively [67].

1.4.3. Hypoglycaemia in children with AI

1.4.3.1. Hypoglycaemia in children with primary AI

In a retrospective study [67], hypoglycaemic seizures and loss of consciousness were reported in 11/102 (10.8%) children with primary AI, with no clear precipitant. All children had classical CAH secondary to 21-OH deficiency. Retrospective data were analysed from the first six years of life. 28 (27.5%) children reported salt wasting crises (seven (25.0%) with hypoglycaemia) and 16 (15.7%) hypoglycaemic episodes without salt loss. 13 (12.7%) children experienced seizures with either hyponatraemia or hypoglycaemia. Most adrenal crises were triggered by infections, often with inappropriate emergency management, but in 11 (10.8%) cases

hypoglycaemia occurred unexpectedly, without evidence of severe illness and without any management errors. Frequency of adrenal crises were reported as 6.5 per 100 patient years (95% confidence interval (CI): 4.6-8.8).

1.4.3.2. Hypoglycaemia in children with secondary AI

Cambiaso et al [65] evaluated the usefulness of continuous glucose monitoring systems (CGMS) to identify nocturnal hypoglycaemia in children with combined ACTH and growth hormone (GH) deficiency. Eleven children aged 1.6-16.8 years were studied. All were on a thrice daily regimen of hydrocortisone in doses of 5.9-10.8mg/ m^2/day. Ten were treated with thyroxine for secondary hypothyroidism, one was treated for gonadotropin deficiency, and none had DI. All children had abnormalities in the hypothalamic-pituitary region on MRI. A CGMS was worn for 36 hours (at least two nights). Glucose measurements of <2.78mmol/L for two consecutive readings were considered a hypoglycaemic event. Nocturnal hypoglycaemia occurred for 1.5% of the time (from 30-155 minutes) in three (27%) children, predominantly in the early hours of the morning and eight (73%) had glucose readings <3.3mmol/L for 5% of the time. Fasting glucose was normal in all children. There were no differences in age or GH treatment between those that had hypoglycaemia and those that did not. However, median hydrocortisone doses were lower in those that experienced hypoglycaemia (5.9 mg/ m^2/day (5.9 – 7.7) versus 8.5 mg/ m^2/day (7.8 – 9.6), p = 0.04) and hydrocortisone dose was significantly related to mean glucose (r = 0.79, p = 0.0035).

1.4.3.3. Hypoglycaemia in both primary and secondary AI in children

Johnstone et al (2008) [66] studied 20 children on two occasions in a randomised cross-over trial: seven had ACTH and GH deficiency; six had primary AI; seven had GH deficiency alone. Children were tested by finger prick monitoring. Blood glucose was tested once at 4am overnight (after six hours fasting), then regularly from 7am to 12pm. No child became hypoglycaemic within 9 hours of fasting. Hypoglycaemia did not occur in children with AI if they received their hydrocortisone medication. If this medication was not received, hypoglycaemia (blood glucose <3mmol/L) occurred in five (25%) patients. Three of seven (4%) children with secondary AI and GH deficiency without treatment and whilst fasted were also hypoglycaemic. One of six (17%) children with primary AI was also hypoglycaemic after

fasting and omitting treatment. One child with GH deficiency alone also became hypoglycaemic.

1.4.4. Hypoglycaemia in the adult population with AI

1.4.4.1. Hypoglycaemia in adults with primary AI

Meyer et al [7] performed CGMS using a blinded MiniMed device (Medtronic, Northridge, CA) on thirteen patients with Addison's disease, five males, eight females with a mean age of 46 years (range 21-71 years). Mean duration of Addison's disease was 13 years (range, 0.25-52 years). Calibration was required three to four times per day. One patient aged 42 years, with a 33-year history of Addison's disease, had an episode of hypoglycaemia (≤2.78mmol/L). He had frequently woken overnight prior to this study but had no other symptoms of hypoglycaemia. He then moved his last dose of hydrocortisone to a later time. Hypoglycaemia resolved, and the patient reported better sleep when the last dose of hydrocortisone was taken later in the day. Data from this study showed that mean nocturnal glucose was no lower than in healthy controls and therefore did not conclude that Addison's disease or its treatment affected glucose metabolism.

1.4.4.2. Hypoglycaemia in adults with secondary AI

Watanabe et al [68] used CGMS (MiniMed CGMS- Gold and iPro2, Medtronic, Northridge, CA) in eleven patients with secondary AI. All patients described occasional morning headache or discomfort. Five patients were excluded from the study, as they experienced, what were described in the manuscript as 'reactive hypoglycaemia', although no definition of this term is given. Patients underwent CGM for 24-48 hours, including at least one night before and after the modification of glucocorticoid replacement regimens in their hospital stay with regular meals (2000 kcal/day). Five out of six patients (83%) had episodes of hypoglycaemia at midnight and in the early morning. Following optimisation of hydrocortisone doses, there were no longer episodes of hypoglycaemia. Mean minimum blood glucose concentrations were higher (3.4 ± 0.28 mmol/L vs 4.6 ± 0.56 mmol/L). However, the mean plasma glucose, mean amplitude of glycaemic excursion and mean maximum plasma glucose showed no difference. All patients had a normal morning fasting glucose.

1.4.5. Hyperglycaemia in paediatric and adult patients with AI

There is very little literature regarding hyperglycaemia in AI. On a search of PubMed using the terms ("Hyperglycaemia" OR "high glucose") AND ("adrenal insufficiency" OR "adrenal" or "congenital adrenal hyperplasia" OR "CAH" OR "Addison's") no articles showing hyperglycaemia in those treated with glucocorticoids for AI were found. There is evidence that high dose or prolonged supra-physiological doses of glucocorticoid therapy is associated with changes in glucose homeostasis, insulin resistance and type 2 diabetes [69]. Insulin resistance in AI may be associated with the effects of over treatment with glucocorticoids. However, to my knowledge there is no evidence that this is associated with hyperglycaemia until a diagnosis of diabetes is made later in life.

1.5. Cardiovascular risk

Cardiovascular disease is a major cause of death. Modifiable risk factors for cardiovascular disease include smoking, poor nutrition, obesity, lack of exercise and hypertension [70]. CAH is the most common cause of AI in childhood, therefore most evidence of cardiovascular outcomes in children with AI are from the population of patients with CAH.

1.5.1. How do we measure cardiovascular risk?

Cardiovascular risk is usually assessed using clinical measures. Adults have used a Framingham risk score for many years. This has progressed to a 30-year Framingham risk score to be used in young adults to predict cardiovascular outcomes over a 30-year period [71, 72]. Risk factors such as sex, BMI, BP, antihypertensive treatment, total and HDL cholesterol, smoking and diabetes have been shown to be significantly related to cardiovascular disease.

Candelino et al describe how cardiovascular risk factors are already detectable in childhood [73]. Some are non-modifiable such as genetics factors and congenital heart disease, whilst others are modifiable such as lifestyle and nutrition. They describe that an early diagnosis is fundamental to ensure optimal life expectancy into adulthood. The most important cardiovascular risk factors in children are thought to be excess weight, arterial hypertension, glucose metabolism and lipid metabolism alterations.

Hypertension can have effects on end organs such as kidneys, heart and vasculature. Hypertension in childhood is associated with increased cardiovascular morbidity into adulthood [74]. Framingham risk score has been shown to be associated with CIMT in healthy adults [75] and adults with human immunodeficiency virus [76]. A meta-analysis in 2012 [77], again in adults showed that CIMT measurement was associated with small improvements in 10- year prediction of a first-time myocardial infarction or stroke but that this improvement was unlikely to be of clinical significance. CIMT has also been shown to be increased in children with traditional risk factors, such as obesity, hypertension, chronic kidney disease, as compared to healthy children [78].

Endothelial dysfunction is also a risk factor for cardiovascular disease development. Endothelial dysfunction is thought to be an early sign of atherosclerotic disease [79]. Flow mediated dilation (FMD) uses ultrasound to assess peripheral artery diameter increases following flow and shear stress after five-minutes of ischaemia. This ischaemia leads to release of nitric oxide from the endothelium stimulating vasodilatation of the artery. It has been shown to strongly and independently predict cardiovascular risk in adults [80, 81]. Normative datasets have been published for children [82].

Von Willebrand factor (VWF) antigen is used to determine the amount of VWF protein present in the blood. VWF activity (also called Ristocetin Cofactor) determines whether the protein is functioning properly. VWF is one of many acute phase reactants. This means that levels will temporarily increase in infections, inflammation, trauma, physical and emotional stress. Levels can also increase in pregnancy or when using oral contraceptives.

VWF is synthesised and stored in endothelial cells. VWF mediates platelet aggregation and adhesion. It has been suggested that high VWF levels can reflect damage to the endothelium or endothelial dysfunction [83, 84]. High levels of VWF have been associated with thrombosis and atherogenesis [83]. Low levels can be associated with von Willebrand's disease, a bleeding disorder.

1.5.2. Cardiovascular and metabolic risk in AI

1.5.2.1. Recent meta-analysis in cardiovascular outcomes in adults and children with CAH

In a recent metanalysis [85] fourteen observational studies (12 longitudinal and two cross-sectional), including 300 children and 137 adults, aged 14 months to 63 years were analysed comparing risk factors for cardiovascular disease and cardiac events in CAH to that of healthy controls matched for BMI, sex and age. Using weighted mean difference and 95% CI, there was an increase in systolic (+4.44mmHg (%5 CI, 3.26 to 5.63 mm Hg) and diastolic (+2.35 mm Hg, 95% CI: 0.49 to 4.2 mm Hg) BP, HOMA-IR (+0.49, 95% CI, 0.02 to 0.96) and CIMT (+0.08 mm Hg, 95% CI: 0.01 to 0.15mm). There was no difference in fasting glucose, insulin or lipids and glucose and insulin concentrations two hours after a glucose load. As cardiac events were sparse, no direct, well-controlled evidence of cardiovascular or metabolic morbidity and mortality associated with CAH was found. However, the authors do note that there are ample increased cardiometabolic risk factors which may predict future cardiac events.

Subgroup analysis in the metanalysis showed that systolic BP was higher only in children and adolescents compared to healthy controls, adults showed no significant difference. The opposite was true regarding diastolic BP, the results were only significantly different in adults but not in children. Fludrocortisone doses were not described in most studies. Children and adolescents were more likely to have a lower fasting blood glucose and higher total cholesterol than healthy controls. Increased CIMT, compared to healthy controls, was more pronounced in adults compared to children. Other planned subgroup analysis included age at diagnosis, sex, genotype, dose of glucocorticoid, type of glucocorticoid, duration of treatment, method of measuring BP and classical versus non-classical CAH were unable to be analysed as there was insufficient data available. Further studies could not be included in the meta-analysis, as they did not provide detailed data for inclusion. These studies have been collated and are summarised in Table 1.3.

Table 1.3: Table showing studies included in the systematic analysis, but without sufficient details to be included in the meta-analysis, Tamhe et al[85]

Publications associated with study	**Demographics**	**Cardiovascular outcomes**
Arlt et al, 2010[86]* Krone et al, 2013[87]* Han et al, 2014 [88]*	- UK - 199 adults (138 women, 65 men) - Median age 34 years (range 18-69 years) - Prospective - Cross sectional study - Glucocorticoid treatment: 26% hydrocortisone, 43% prednisolone, 19% dexamethasone, 10% combination	- Higher rates of hypertension in patients diagnosed after 1 year, compared to those diagnosed early - Hypertension was higher in those treated with glucocorticoids only and did not receive mineralocorticoids - No differences in BP between genetic mutations - Women with classical CAH had higher DBP
Falhammer et al, 2015 [89]	- Sweden - 558 patients with CAH compared to 58,800 controls matched for sex, age and birthplace - National register - Median age 26 years (range 0-92 years)	- CAH had increased frequency of hypertension, hyperlipidaemia, atrial fibrillation, venous thromboembolism, obesity and diabetes compared to healthy controls
Finkielstain et al, 2012 [90]	- US - Patients from 36 states, Puerto Rico and nine patients from other countries - Cross-sectional study	- Elevated BP was more common in patients with classical CAH compared to non-classical CAH

	- 170 children (aged 6month-17 years) and 74 adults (18-68 years)	
Bonfig et al, 2016 [91]	- Germany - 716 children and adolescents - Aged 3-18 years	- Overall prevalence of hypertension (>95th centile) 12.5% - Prevalence of hypertension was higher in younger children than adolescents (18.5% vs 4.9%) - BP was higher in salt wasting than simple virilising CAH - Until 8 years of age, fludrocortisone dose but not hydrocortisone dose correlated significantly with BP
Moreira et al, 2012 [92]	- Brazil - Case-control study - 68 adults (aged 28.4 ± 9 years) - Treated with dexamethasone	- Hypertension was found in 12% of patients, and heterozygotes for the B*cl*I polymorphism with intron 2 of the glucocorticoid receptor (NR3C1) gene exhibited higher systolic BP than wild-type subjects
Volkl et al, 2006 [93]	- Cross-sectional retrospective study - 89 children and adolescents - 48 females, 41 males - Aged 0.2-17.9 years - All with classical CAH, confirmed molecular genetic analysis, on glucocorticoids ± mineralocorticoids	- Daytime and night-time systolic BP were significantly elevated - Daytime diastolic BP was lower and normal overnight - Overall, there was a normal nocturnal drop in systolic BP but not in diastolic BP

UK: United Kingdom, CAH: congenital adrenal hyperplasia, BP: blood pressure.
*All manuscripts and findings associated with same cohort

Whilst writing European clinical guidelines [94], the above systematic review was commissioned. Following these findings, the guideline suggests that lifestyle counselling should begin from an early age in children with CAH, in view of the increased surrogate markers of cardiovascular disease that can be found in the population.

1.5.2.2. Further cardiovascular outcomes in children with CAH

Increased CIMT has been reported in many studies of children with CAH [95-99]. In children, administration of the highest dose of hydrocortisone in the evening can be associated with increased 24-hour BP without improved control of androgen concentrations [100].

1.5.2.3. Further cardiovascular outcome in adults with CAH

Increased prevalence of insulin sensitivity, hypertension and obesity is reported in adults with CAH [86, 90, 101-105]. Both higher BP and absent nocturnal BP dipping has been reported in CAH in both adults and children [101, 103]. In adults, it has been speculated that exposure to excess glucocorticoids from an early age may account for increased risk for cardiovascular mortality [13].

1.5.2.4. Metabolic risk in patients with CAH

Patients with AI may also experience poor metabolic health. High cortisol concentrations are associated with obesity, abdominal adiposity and diabetes mellitus [106]. Loss of the cortisol circadian rhythm is associated with obesity, diabetes mellitus and increased biomarkers of cardiovascular disease [58].

It is known that children with CAH may have increased fat mass [107] and are at a higher risk of being overweight or obese. [91, 101, 108-110]. It has been speculated that this could be secondary to glucocorticoid dose [108, 111]. Adiposity rebound is the second rise in BMI that usually happens within the first three to seven years of life. It is thought that an early adiposity rebound can be a risk factor for obesity later in life [112], and this phenomenon has been described in children with CAH [113].

Impaired insulin sensitivity is seen in children and young people with CAH. This has also been speculated to be related to glucocorticoid treatment [4, 103, 114].

In an adult CAH cross-sectional study [86], 41% were obese, 46% had hypercholesterolaemia and 29% had insulin resistance. Subjective health status was also significantly impaired.

1.5.2.5. What is not yet known regarding cardiovascular disease in CAH in children?

Based on this literature, it is widely known that there is an increased incidence of cardiovascular disease in patients with CAH. Further knowledge regarding the reason for this is required. There is speculation that this may be associated with higher doses of glucocorticoid use, that this may be secondary to the non-physiological diurnal pattern of cortisol exposure, increased androgens or other confounding factors. Endothelial dysfunction, also a known predictor of cardiovascular disease, has not been reported in adults or children with CAH. Alongside this, interventions used to reduce this risk must be assessed.

1.5.2.6. Cardiovascular and metabolic risk in Addison's disease

There are very little data addressing cardiovascular risk factors in children and adolescents with Addison's disease, most likely because this is a rare diagnosis in the paediatric population.

In adults, Addison's disease has been associated with an increased risk ratio for all-cause mortality, mainly due to cardiovascular death. It was proposed that this was secondary to excess glucocorticoid exposure [57]. Further studies confirm increased cardiovascular risk in Addison's disease [115]. This risk was much higher in female patients compared to males, and in those treated with higher hydrocortisone and fludrocortisone doses. In a population of Swedish and South African adults with Addison's disease 20% of both cohorts had hypertension and diabetes mellitus [116]. South African patients with Addison's disease had higher lipid profiles than Swedish patients ($p<0.001$) even though Swedish patients were on higher total daily doses of hydrocortisone ($p<0.001$). The differences between the two populations could not be explained.

Hypertension can be difficult to manage in Addison's disease. It is recommended, in adults, that a vasodilator should be used to treat hypertension in primary AI, particularly Addison's disease rather than stopping mineralocorticoid replacement, although it is stated that a dose reduction should be considered [117]. Glucocorticoid doses should also be optimised in such cases [118].

1.5.2.7. Cardiovascular and metabolic risk in both primary and secondary AI

Ngaosuwan et al [119] performed a retrospective cohort study in the UK, using the UK general practitioner database. 6821 adult patients with AI (2025 with primary AI, 3948 with secondary AI) were compared to 67, 564 matched records. Hazard ratios (95% CI) for cardiovascular events in patients with AI of any cause was 1.28 (1.2-1.36, unadjusted for diabetes, hypertension, dyslipidaemia, previous cardiovascular disease and smoking) and 1.07 (1.01-1.14, adjusted for the above). Increased cerebrovascular events in patients with secondary AI were reported (1.53 (1.34-1.74, adjusted)) and were associated with cranial irradiation. Cardiovascular mortality data were available for 3547 patients and 34,944 controls. The adjusted hazard ratio for ischaemic heart disease mortality was 1.86 (1.25-2078) for primary AI and 1.39 (1.02-1.89) for secondary AI. Co-morbidities were thought to account for the increased cardiovascular events. However, cerebrovascular disease was independently increased in secondary AI, associated with irradiation. Even after adjusting for co-morbidities there was an increased cardiovascular mortality in both primary and secondary AI.

1.5.2.8. Cardiovascular and metabolic risk associated with secondary AI

In secondary AI, it was found that using glucocorticoid doses of >20mg/ m^2/day led to a higher prevalence of metabolic risk factors, leading to an increased risk of cardiovascular disease [120]. It is speculated that glucocorticoid replacement can lead to obesity, hypertension, diabetes and hyperlipoproteinaemia [10-12]. In hypopituitary patients, females seem to be at a higher risk of metabolic syndrome than males [121]. Studies have shown that lower doses of hydrocortisone in male adults with secondary AI can improve arterial stiffness index, as well as allow a more physiological nocturnal dip in BP [122]. Higher doses of glucocorticoids are associated with endothelial dysfunction in secondary AI [123].

1.5.2.9. Role of mineralocorticoids in cardiovascular risk in primary AI

Alongside excess doses of glucocorticoids, excessively high doses of fludrocortisone are likely to be associated with hypertension. Hypertension can be seen in primary aldosteronism and 11β-hydroxylase deficiency, a rare form of CAH associated with mineralocorticoid excess.

1.6. Summary

Within this introduction I have discussed normal physiology associated with the HPA axis before moving on to assess pathological processes that can lead to hypocortisolaemia, both at the level of the hypothalamus and pituitary (secondary AI) and the level of the adrenal gland (primary AI). International guidance exists for diagnosis, management and care of patients with AI.

AI is treated with glucocorticoid preparations that cannot mimic the diurnal release of cortisol accurately. AI has been described to be associated with hypoglycaemia, particularly in the adult population. This may be secondary to troughs of cortisol concentrations pre-dose. Hypoglycaemia can be associated with early morning headaches and poor concentration. Hyperglycaemia has not been described until the onset of diabetes as an adult, although AI is associated with an increase in insulin resistance.

Morbidity and mortality in AI are higher than the background population. This is thought to be secondary to cardiovascular disease, alongside the risk of adrenal crisis. AI has been described to be associated with increased BMI, hypertension, increase insulin resistance and increased CIMT. This has been described in both adults and children. CAH is the most common form of AI in children, other than iatrogenic effect of exogenous steroids. CAH is therefore the most described population of AI, particularly in relation to cardiovascular disease.

Within this thesis I aim to assess the population with CAH in Alder Hey Children's NHS foundation trust, a tertiary hospital, comparing their care against international guidelines; assess salivary concentrations of cortisol, cortisone and other adrenal biomarkers in healthy children and young people before moving on to assess both glucose regulation and cardiovascular health in children with primary AI (GRACE1) and secondary AI (GRACE 2). Salivary hormone concentrations in children with AI will be compared to the dataset from healthy children. These studies were set up during the COVID-19 pandemic. As there was uncertainty regarding recruitment, particularly of non-COVID related research, I also analysed results of LDSSTs performed locally for the diagnosis of AI. These data are described within this thesis.

2. Chapter 2: Assessment of local practice of management of CAH compared to international guidance

2.1. Introduction

As part of a feasibility assessment, prior to study recruitment for GRACE 1 (see chapter 4), management of children with CAH at Alder Hey Children's hospital was audited against international practice guidelines from the Endocrine Society, United States of America [94]. The majority of patients with CAH attending Alder Hey have 21-OH deficiency. No internationally recognised guidelines are available for the rarer forms of CAH and therefore this guideline was applied for all patients.

2.2. Methods

Data were collected from electronic records and clinic letters between March 2020 and April 2021, coinciding with COVID-19 pandemic. In the year preceding data collection there had been disruption to usual hospital services, secondary to COVID restrictions. Recommendations for the management of adults with CAH were not relevant to this population and therefore have been excluded from the audit. This audit was registered with Alder Hey Children's audit department (audit number 6321).

2.3. Results

There were 38 children and young people with CAH cared for at Alder Hey at the time of the audit: 36 patients had 21-OH deficiency of whom 33 were classed as 'growing individuals' and three were infants. Two patients had non-classical CAH, one had 3β-HSD deficiency and one had 11β-HSD deficiency.

2.3.1. New born screening

Recommendation 1.1: We recommend that all new born screening programs incorporate screening for CAH due to 21OH deficiency.

The UK do not currently perform testing for CAH and therefore this section was excluded from analysis.

2.3.2. Prenatal treatment of CAH

Recommendation 2.1: We advise that clinicians continue to regard pre-natal therapy as experimental. Thus, we do not recommend specific treatment protocols

We did not have access to maternal medical health records, therefore this was excluded from this analysis.

2.3.3. Diagnosis

Recommendation 3.2: In symptomatic individuals past-infancy, we recommend screening with an early morning (before 8am) baseline serum 17OHP measured by LC-MS/MS.

Recommendation 3.3: In individuals with borderline 17OHP concentrations, we recommend obtaining a complete adrenocortical profile after cosyntropin stimulation test to differentiate 21OH deficiency from other enzyme defects

Recommendation 3.4: In individuals with CAH, we suggest genotyping only when results of the adrenocortical profile after a cosyntropin stimulation test are equivocal, or cosyntropin stimulation cannot be accurately preformed (i.e., patient receiving glucocorticoid), or for purposes of genetic counselling.

All children had at least one biochemical test confirming their diagnosis, including pre-8am 17OHP, SDSST test with 17OHP stimulation or genotyping. Genotyping is not a requirement for diagnosis, as per the guidelines, if the diagnosis has been confirmed with 17OHP or Synacthen testing. However, a genotype was available for all growing individuals as this had been used to aid genetic counselling for patients and families. Two of the three infants had not yet been genotyped, but this was being processed at the time of this audit.

2.3.4. Treatment of classic and non-classical CAH

Recommendation 4.1: In growing individuals with classic CAH, we recommend maintenance therapy with hydrocortisone

Recommendation 4.2: In growing individuals with CAH, we recommend against the use of oral hydrocortisone suspension and against the chronic use of long-acting potent glucocorticoids

Recommendation 4.3: In the new born and early infancy, we recommend using fludrocortisone and sodium chloride supplements in the treatment regime

Recommendation 4.5: In all individuals with classic CAH, we recommend monitoring for signs of glucocorticoid excess, as well as for signs of inadequate androgen normalisation, to optimise the adrenal steroid profile

Recommendation 4.6: In all individuals with classic CAH, we recommend monitoring for signs of mineralocorticoid deficiency or excess

Recommendation 5.1: In children and adolescents with inappropriately early onset and rapid progression of pubarche or bone age and in adolescent patients with overt virilization we suggest glucocorticoid treatment of non-classical CAH.

Recommendation 5.2: In asymptomatic nonpregnant individuals with non-classical congenital adrenal hyperplasia we recommend against glucocorticoid treatment

All children were receiving appropriate treatment. Those with salt-losing CAH were treated with glucocorticoids, mineralocorticoids and where necessary, salt supplementation. Those with simple virilising CAH were treated with glucocorticoid only. Children with non-classical CAH were, appropriately, not receiving any of these medications.

Some children were using oral solution hydrocortisone, some Alkindi® granules, some tablet SF-HC and others were using Plenadren®.

2.3.5. Stress dosing

Recommendation 4.7: In all patients with CAH who require glucocorticoid treatment, for situations such as febrile illness (>38.5ºC), gastroenteritis with dehydration, major surgery accompanied by general anaesthesia, and major trauma we recommend increasing the glucocorticoid dosage.

Recommendation 4.8: In patients with CAH under every day mental and emotional stress and minor illness and/or before routine physical exercise we recommend against the use of increased glucocorticoid doses

Recommendation 4.9: In patients with CAH who require treatment, we recommend always wearing or carrying medical identification indicating that they have adrenal insufficiency

Recommendation 4.10: In patients with CAH, we recommend educating patients and their guardians and close contact on adrenal crisis prevention and increasing the dose of glucocorticoid (but not mineralocorticoid) during intercurrent illness

Recommendation 4.11: We recommend equipping every patient with CAH with a glucocorticoid injection kit for emergency use and providing education on parenteral self-administration or parental administration of emergency glucocorticoids.

Documentation of children receiving an emergency glucocorticoid kit was almost 100%. Documentation of education regarding its use was discussed in 60% of children within the year prior to data collection. This included a discussion regarding increased glucocorticoid

treatment doses during periods of illness. Sick day doses were included in almost 100% of clinic letters and 100% of health care plans that were shared with the family. There was no evidence that this was prescribed for use in mental and emotional stress. This is not practiced locally. The use of medical ID was only documented in one child.

2.3.6. Monitoring

Recommendation 4.12: In patients ≤ 18 months with CAH, we recommend close monitoring in the first 3 months of life and every 3 months thereafter. After 18 months, we recommend evaluation every 4 months.

Recommendation 4.13: In paediatric patients with CAH, we recommend conducting regular assessment of growth velocity, weight, blood pressure, as well as physical examination in addition to obtaining biochemical measurements to assess the adequacy of glucocorticoid and mineralocorticoid.

Recommendation 4.14: In paediatric patients with CAH over the age of 2 years, we advise annual bone assessment under near-adult height is attained.

Biochemical monitoring at Alder Hey is undertaken by measurement of blood spot 17OHP at multiple time points through the day, timed against doses of glucocorticoid. Blood spot cards are sent to families, blood spots are collected at home and returned by post. Despite the pandemic, 90% of patients had completed a blood profile within the last year.

Once a year, patients attend for a venous blood sample to be collected for measurement of electrolytes, resting plasma renin activity, aldosterone, ACTH, 17OHP, A4, DHEAS and testosterone. In girls ≥ 8 years and boys ≥ fasting lipids, glucose, insulin and c-peptide are performed. At the same appointment, specialist nurses review parents', and where appropriate, patients' knowledge of CAH, sick day rules etc. This appointment had been completed in 60% of all patients, but only in one of the three (33%) infants. Seventy percent of patients had been reviewed at least four-monthly via face to face or telephone consultation. An annual bone age had only been performed in two (5%) children.

Height, weight and growth velocity has been measured in all children. Examination was documented in approximately 90%. BP was only recorded within the last year in 40% of children.

2.3.7. Transition to adult care

Recommendation 6.1: In adolescent patients with CAH, we suggest that the transition to adult care begins several years prior to dismissal from paediatric endocrinology.

Recommendation 6.2: In adolescent females with congenital adrenal hyperplasia, we suggest a gynaecological history and examination to ensure functional female anatomy without vaginal stenosis or abnormalities in menstruation.

In Alder Hey Children's NHS foundation trust, transition discussions are advised to be initiated at approximately 13 years of age. Eight of the 33 patients with CAH were eligible to begin discussions around transition. This was documented in 50% of these cases and 40% had been reviewed in joint clinics with an adult physician. 100% of females eligible for transition discussions had met with an adult gynaecologist.

2.3.8. Surveillance for long term complication of CAH and its treatment

Recommendation 6.10: For patients with CAH, we suggesting introducing counselling regarding healthy lifestyle choices at an early age to maintain BMI within the normal range to avoid metabolic syndrome and related sequelae

Recommendation 6.13: In males with classic CAH, recommend periodic testicular ultrasound to assess for the development of testicular adrenal rest tumours

Recommendation 6.14: In patients with CAH, we recommend against routine evaluation for cardiac and metabolic disease beyond that recommended in the general population

There was no specific documentation found regarding healthy lifestyle choices. BMI was documented at each consultation. No testicular ultrasounds were performed on males with CAH. Only participants of GRACE 1 (see chapter 4) had evaluation of cardiac and metabolic disease above that recommended for the general population.

2.3.9. Surgery

Recommendation 7.1: In all paediatric patients with congenital adrenal hyperplasia, particularly minimally virilised girls, we advise that parents be informed about surgical options, including delaying surgery and/or observation until the child is older

Recommendation 7.2: In severely virilised females, we advise discussion about early surgery to repair the urogenital sinus.

Recommendation 7.3: In the treatment of minors with congenital adrenal hyperplasia, we advise that all surgical decisions remain the prerogative of families in joint decision making with experienced surgical consultants

Recommendation 7.4: In female patients with congenital adrenal hyperplasia for whom surgery is chosen, we suggest vaginoplasty using urogenital mobilization and, when chosen, neurovascular-sparing clitoroplasty for severe clitoromegaly.

Thirteen of the growing individuals were female, three of whom had undergone surgery. Surgery had been discussed and joint decision making had been made with all families within the DSD multi-disciplinary team.

2.3.10. Adrenalectomy

Recommendation 8.2: In patients with CAH, we recommend that adrenalectomy is not performed.

No patients had undergone an adrenalectomy.

2.3.11. Mental health

Recommendation 9.1: For individuals with congenital adrenal hyperplasia and their parents, we recommend behavioural/mental health consultation and evaluation to address any concerns related to congenital adrenal hyperplasia

50% of growing individuals had evidence of referral to psychological services. Almost all children had been referred for genetic counselling.

2.4. Discussion

2.4.1. What are we doing well?

All diagnoses are supported by appropriate biochemical and genetic testing. All patients are receiving appropriate treatment for their underlying diagnosis (salt wasting, simple virilising or non-classical). This audit highlights what treatment children were receiving but doses were not collected. All children with CAH have evidence of receiving an emergency glucocorticoid kit. All females with CAH have met a consultant gynaecologist. Surgical discussions are occurring in those children where it is appropriate. Multi-disciplinary DSD input has occurred in all children for whom it was recommended. Appropriately, no child had an adrenalectomy. These data were collected for the time period between March 2020 and April 2021, during the COVID-19 pandemic. In light of this, monitoring and regular assessments have been reasonably good.

2.4.2. Key issues

No children received new born screening. This has not been adopted within the UK and therefore not something that we can impact locally. Not all patients had been reviewed every four months. This was difficult during the period monitored. Many changes took places within the hospital secondary to the COVID pandemic restrictions. Movement to virtual and telephone consultations increased during this time. The service aims to provide four-monthly appointments for patients with CAH. This would be useful to reassess now that restrictions have settled. We now alternate face to face and telephone consultations in some patients. It would be interesting to see if this has increased appointment uptake for patients who travel further.

Bone age has not been performed annually in most. It has been assessed in only two (5%) patients within the year of assessment. Clinically, symptoms, signs and auxology parameters will usually highlight over or under treatment before bone age delay or advancement will be seen. However, this is recommended within the guideline and would serve to support clinical assessment.

BP is not recorded at every visit and was only documented within the preceding year in 40% of children. BP is particularly important in children and young people treated with fludrocortisone and can guide treatment.

Discussions surrounding medical ID have not been documented. This may have been discussed but as there is no evidence this could not be assessed. 60% of children had discussed emergency hydrocortisone management. This is consistent with the number of children who had attended their annual review appointment.

Not all patients have been offered a referral to psychological services. This is particularly true of male children who would not be routinely seen as part of the DSD service. Plans for transition to adult services are not documented in all cases.

2.4.3. Recommendations

It is recommended that an annual review proforma is updated to include those key areas to ensure that they are assessed regularly including: BP measurements, documentation of

medical ID, updates on emergency care management and assessment of whether psychological input is required. The guidelines have used terms such as ‘regular assessment’. Locally, a decision has been taken that children should be seen four to six-monthly depending on clinical need.

2.5. Re-audit

The findings of this audit have been presented locally and the changes to the service, noted above, have been implemented. The service will be re-audited in one to two years.

3. Chapter 3: Salivary biomarkers in healthy children (SMILE) – analysis of adrenal biomarkers

3.1. Introduction

Measurements of hormones in saliva has been recognised for more than fifty years. Recent developments in sample analysis have generated renewed interest in the use of saliva for the diagnosis of conditions of cortisol excess [124] and deficiency [125], and adequacy of hydrocortisone replacement therapy [126]. More recently, data reporting the concentrations of other salivary adrenal biomarkers have been reported, including 17OHP, A4 [127], testosterone, 11KT and 11OHA4 [128]. These hormones are of interest for monitoring patients with CAH, particularly relating to assessment of over and undertreatment, and other disorders of the adrenal gland.

Measurement of salivary hormone concentrations in children has a number of advantages over serum. Only free, biologically active hormone is measured. Samples can be collected during normal daily activities at home or in school. Stress induced rises in cortisol induced by hospital visits and blood tests are avoided. Furthermore, there will be fewer lost days from school and work. There are also potential healthcare cost savings. Previous literature support that salivary steroids may be stable at room temperature for up to five days to facilitate this [127].

Cortisol activity is regulated at a tissue level by two isoforms of the enzyme 11β hydroxysteroid dehydrogenase (11βHSD). Cortisol is inactivated by conversion to cortisone by 11βHSD type 2, and generated from cortisone by 11βHSD type 1. It is unknown as to whether there are similar enzymes at the level of the salivary gland that catalyse the other salivary adrenal biomarkers. Ninety to 95% of serum steroids circulate bound to proteins and only 5-10% is unbound or 'free' and biologically active. Free, but not bound, steroids can diffuse through the parotid gland. Measurement of steroids by liquid chromatography tandem mass spectrometry (LC-MS/MS) is the preferential option to measure both serum and salivary steroids [94]. A4 and 17OHP are present in low levels in saliva making LC-MS/MS even more desirable.

It is recommended that A4 and 17OHP are monitored in treatment of adrenal disease particularly CAH [129]. Normal A4 concentrations for age and sex are considered as a marker of good control in CAH, whilst normal or suppressed 17OHP concentrations may show overtreatment. There is a strong correlation between concentrations of A4 and 17OHP measured in saliva and serum [128-130]. As patients with CAH take hydrocortisone at several time points throughout the day, salivary steroid marker measurement at similar time points may be beneficial to guide treatment.

11-oxygenated 19-carbon (11oxC19) are adrenal derived steroids and include 11KT, 11β-hydroxy testosterone (11OHT), 11-ketoandrostenedione (11KA4) and 11OHA4. The formation of 11oxC19 steroids can be seen in Figure 3-1. The adrenal gland was thought to be a source of weak androgens including DHEA, DHEAS and A4, precursors for testosterone and dihydrotestosterone which were thought to be the most potent androgens.

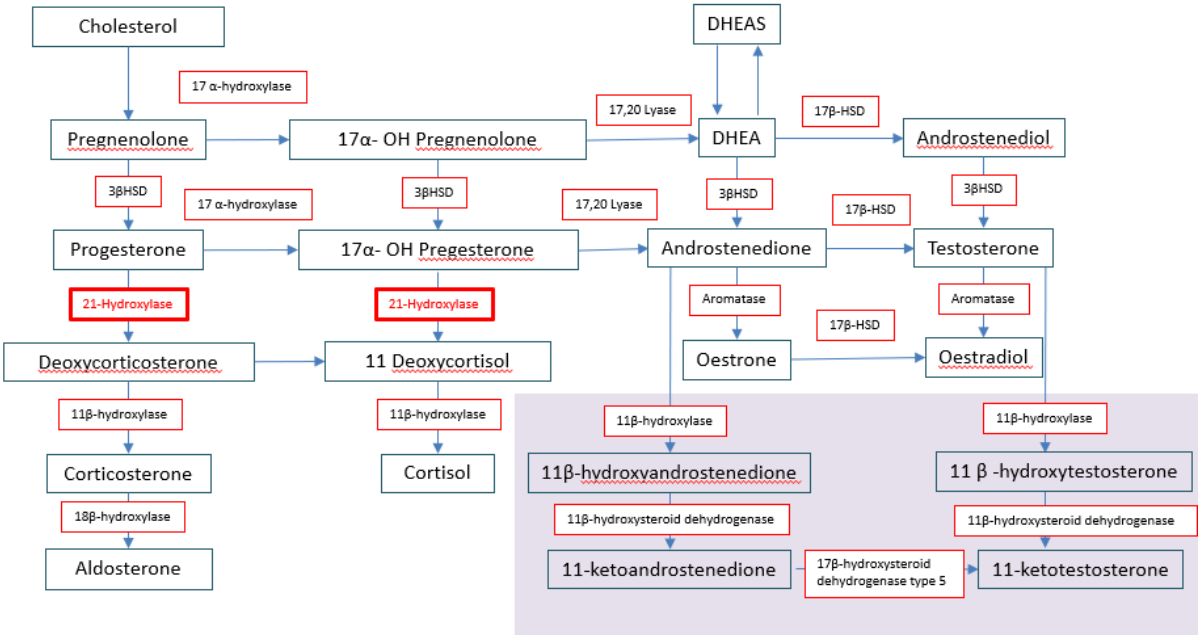


Figure 3-1: Figure showing steroidogenesis including the production of 11oxC19 steroids. HSD: hydroxysteroid dehydrogenase. Purple shading shows 11oxC19 component of pathway, adapted from [131] and [132].

11oxC19 steroids are synthesized from A4 and testosterone by the enzyme cytochrome P450 11β-hydroxylase (CYP11β1) which is found predominantly in the adrenal cortex. It was initially hypothesized that these steroids were the product of testicular Leydig cells and ovarian theca cells. However, CYP11β1 activity is insignificant in these cells compared to the adrenal cortex. 11KT and 11- dihydrotestosterone (11DHT) are thought to be potent agonist of the human

androgen receptor, with a similar potency to testosterone and dihydrotestosterone [133-135]. 11KT concentrations in peripheral serum and adrenal vein sampling are similar in men and women, whilst testosterone is higher in men, also suggesting that gonadal derived testosterone is not an important source of 11OHT and 11KT.

Recent evidence has highlighted that 11oxC19 adrenal androgens may have a place in the diagnosis and monitoring of conditions such as: CAH, particularly 21-OH deficiency; PCOS; prostate cancer and premature adrenarche [136]. The four 11oxC19 steroids (11KT, 11OHT, 11KA4 and 11OHA4) were reported to be 3-4 times higher in serum in patients with CAH [137], in a cross-sectional study of 38 (19 male) patients with classical CAH secondary to 21-OH deficiency, aged 3-59 years, compared to 38 age and sex matched controls [137].

In 21-OH deficiency, low cortisol leads to a loss of negative feedback at the level of the hypothalamus and pituitary and a rise in ACTH. This rise in ACTH stimulates the adrenal gland to synthesis and release androgens. However, it has been shown that DHEA and DHEAS are often uncharacteristically low in patients with 21-OH deficiency [137, 138].

An alternative pathway of steroid metabolism, known as the “backdoor” pathway, has also been described [139]. Kamrath et al describe increased production of androsterone, an alternative metabolite in those with 21-OH deficiency compared to controls. The urinary excretion of 11OHA4 (a metabolite of androsterone) was highly variable. Urine metabolites of both the classic and alternative or “backdoor” pathway were raised in urine in patients on modified release and conventional glucocorticoids. They also showed diurnal excretion [140].

A4 and, in women, testosterone concentrations are routinely used as biomarkers of the adequacy of treatment of 21-OH deficiency. However, these steroids correlate poorly with clinical findings of androgen excess [141, 142]. A4 and testosterone are synthesised in both the gonads and the adrenal gland, which may explain their poor clinical utility. Turcu et al found that all four 11oxC19 steroids correlated with adrenal volume on CT scans in adult patients with CAH [143]. They also found that 11oxC19 steroids were higher in males with testicular adrenal rest tumours, an indicator of poor control of CAH, compared to men without. Poorly controlled post pubertal males with 21-OH deficiency may have a rise in 11KT, leading to gonadal suppression and poor testicular production of testosterone. 11KT:

testosterone ratio may be a good clinical indicator of disease control, particularly in post pubertal males. 11KT correlates with testosterone in women and correlates inversely in post pubertal males [131]. This was true of 114 patients with classical CAH, aged 2-67 years (median age 15 years, with 59% of patients younger than 18 years). There was a direct correlation between testosterone and all four 11oxC19 steroids in girls at all tanner stages of puberty. 11KT and 11OHA4 also correlate positively with testosterone in boys with tanner stage 1-2 but correlated negatively in those who were Tanner stage 5.

To my knowledge, there are few data describing salivary adrenal biomarker concentrations (including A4, testosterone, 11KT and 11OHA4) in healthy children. Recent literature [127] showed diurnal concentrations of both 17OHP and A4 in saliva in both adults and children. Pre-pubertal and adult reference ranges were determined. The population were aged 4-75 years and samples from 251 participants were included for analysis. Saliva was collected via passive drool between 7-8am, 2-3pm and 10-11pm. Participants were excluded if they were receiving glucocorticoid treatment, were pregnant, aged less than four years or worked night shifts. Analysis of pre-pubertal children and adults aged 16 years and over were described. Boys aged 11-15 years and girls aged 10-15 years were excluded as pubertal status was not recorded. In both children and adults, there were no difference between 17OHP and A4 in males compared to females. A4 and 17OHP increased up to the age of 30 years, plateaued, then decreased from 40 years of age. All ages showed decreasing concentrations of both biomarkers throughout the day. Salivary concentrations of both A4 and 17OHP were more than tenfold lower than serum measurements, as anticipated as only free hormone is measured in saliva, in contrast to measurements made in serum, which include both protein-bound and free hormone. Concentrations of both A4 and 17OHP were higher in adults compared to children. This may be explained by the production of gonadal A4 and 17OHP from the testosterone and oestrogen, which are higher in adults than in prepubertal children. Serum and salivary steroid hormones, including 17OHP and A4 have variably been associated with age, sex, pubertal development, menstrual cycle and BMI [127, 144, 145].

3.2. Methodology

3.2.1. Aim

1. To report salivary cortisol and cortisone concentrations throughout the day in healthy children and young people, building on previous work [146], with a larger cohort.

2. To define reference intervals for salivary adrenal biomarkers (testosterone, A4, 11-KT and 11OHA4) in healthy children and young people.

3.2.2. Objectives

To describe:

1. Salivary adrenal androgen biomarker concentrations 30 minutes after waking.
2. salivary cortisol, cortisone and adrenal androgen biomarker concentrations measured every two hours during waking hours.
3. mean salivary adrenal androgen biomarkers during waking hours.
4. the frequency of samples in which salivary cortisol, cortisone and other adrenal androgen biomarkers are below detectable limits.

To further explore relationships between salivary biomarkers and the following parameters:

1. Age
2. Gender

3.2.3. Recruitment

Recruitment took place as part of an MPhil project performed by Orla Bright. I supported the application to the ethical committee, preparation of the participant information leaflets and consent forms, logistical help with salivary sampling and supported the consent process for this study. I have performed all the analyses in this thesis and they are not reported elsewhere.

Healthy children and young people were approached if they were: siblings of patients attending hospital; children of staff working at Alder Hey Children's hospital or children attending Alder Hey for treatment for a condition that does not affect, or is associated with, an abnormality of adrenal function, for examples children attending for insertion of grommets.

Parents were approached in the outpatient setting, through communication supported by Alder Hey Trust communications team, or whilst attending a pre-operative appointment.

3.2.4. Inclusion criteria

1. Children and young people aged 5 – 18 years.

3.2.5. Exclusion criteria

1. Children with oral conditions likely to results in blood contamination of saliva samples including gingivitis, mouth ulcers and those undergoing dental procedures.
2. Children with conditions likely to affect serum cortisol or CBG including abnormalities of thyroid or anterior pituitary hormone secretion, psychiatric pathology, type I or type 2 diabetes, cystic fibrosis, protein losing enteropathies, nephrotic syndrome and patients undergoing renal dialysis.
3. Children with a family history of adrenal insufficiency due to an inherited condition.
4. Children receiving medications likely to affect serum cortisol or CBG including glucocorticoids, sex steroids, thyroxine, growth hormone, azole compounds, insulin and metformin.
5. Children below the age of five years (because of concerns that the cotton wool roll used to collect saliva in the Salivette device may present a choking hazard in young children).

3.2.6. Study visit

3.2.6.1. Characteristics

Participants attended the clinical research facility (CRF). Written, informed consent and assent was obtained. Information was taken regarding age, sex and post code to determine index of multiple deprivation decile. Participants were measured on electronic scales and measured on a calibrated stadiometer. BP was measured using automated BP cuffs on three occasions, one minute apart. An average BP over the three readings was recorded. SDS for height and BMI were derived from reference data taking into account height and gender [147]. BP centiles were determined according to age, sex and height [148].

3.2.6.2. Salivary sampling

Participants were given salivary sampling collection kits with salivettes (*Salimetrics®, New Market*). Participants were asked to take the first sample 30 minutes after waking, followed by two-hourly sampling throughout the day. These samples were then returned to the CRF with a booklet informing of the time woken, whether the participant woke spontaneously or were woken by an alarm or person. Sample timings were also recorded. Saliva was measured for cortisol, cortisone, testosterone, A4, 11KT and 11OHA4 using LC-MS/MS analysis using a Waters Xevo TQS micromass spectrometer and a Waters Acquity iclass LC system with an

electrospray source operated in positive ionization mode following sample preparation by protein precipitation.

3.3. Results

3.3.1. Salivary cortisol and cortisone

85 (48 male) participants with a median age 11.0 years (range 5-18 years). Early morning salivary cortisol was available in 83 participants with a median concentration of 8.7nmol/L (5^{th}-95^{th} centile 2.9-16.9nmol/L). These values decreased throughout the day and can be seen in Table 3.1.

Table 3.1: Table showing number of samples and median (5th -95th centile) salivary cortisol concentrations throughout the day in healthy children

Time after waking	Number of sufficient samples	Salivary cortisol (nmol/L), Median (5^{th} – 95^{th} centile)	Number (%) of samples salivary cortisol undetectable (<0.3nmol/L)
30 minutes	83	8.7 (2.9 – 16.9)	0 (0.0%)
2 hours	82	2.2 (0.9 – 9.7)	1 (1.0%)
4 hours	85	2.0 (0.6 – 4.0)	1 (1.0%)
6 hours	85	1.5 (0.5 – 5.4)	1 (1.0%)
8 hours	82	1.3 (0.5 – 5.5)	4 (4.9%)
10 hours	82	0.7 (0.2 – 3.0)	11 (13.4%)
12 hours	70	0.4 (0.2 – 2.4)	24 (34.3%)

nmol/L: nanomole per litre.

Salivary cortisone was available in 83 participants with a median concentration 32.4 nmol/L (5^{th} – 95^{th} centile 18.0 – 52.8nmol/L), again this decreased throughout the day (Table 3.2).

Table 3.2: Table showing number of samples and median (5th -95th centile) salivary cortisone concentrations throughout the day in healthy children

Time after waking	Number of sufficient samples	Salivary cortisone (nmol/L), Median (5th – 95th centile)	Number (%) of samples salivary cortisone undetectable (<0.3nmol/L)
30 minutes	83	32.4 (18.0 – 52.8)	0 (0%)
2 hours	82	15.3 (8.0 – 34.0)	0 (0%)
4 hours	85	13.4 (8.2 – 26.7)	0 (0%)
6 hours	85	12.1 (6.3 – 28.1)	0 (0%)
8 hours	82	10.4 (4.8 – 27.9)	0 (0%)
10 hours	83	7.1 (3.5 – 19.7)	0 (0%)
12 hours	71	3.7 (2.0 – 10.5)	0 (0%)

nmol/L: nanomole per litre.

The salivary cortisone: cortisol ratio was 4.1 thirty minutes after wakening and rises throughout the day (Table 3.3).

Table 3.3:Table showing number of samples and median (5th -95th centile) salivary cortisone: cortisol ratio throughout the day in healthy children

Time after waking	Salivary cortisone: cortisol ratio, median (5th – 95th centile)
30 minutes	4.1 (2.2 – 7.0)
2 hours	7.1 (2.7 – 11.7)
4 hours	7.2 (3.9 – 16.4)
6 hours	8.4 3.4 – 16.8)
8 hours	8.0 (3.6 – 15.7)
10 hours	9.9 (3.4 – 16.7)
12 hours	7.6 (1.0 – 15.7)

Median and 95th centiles of salivary cortisol and cortisone concentrations over the day are shown in Figure 3-2 and Figure 3-3.

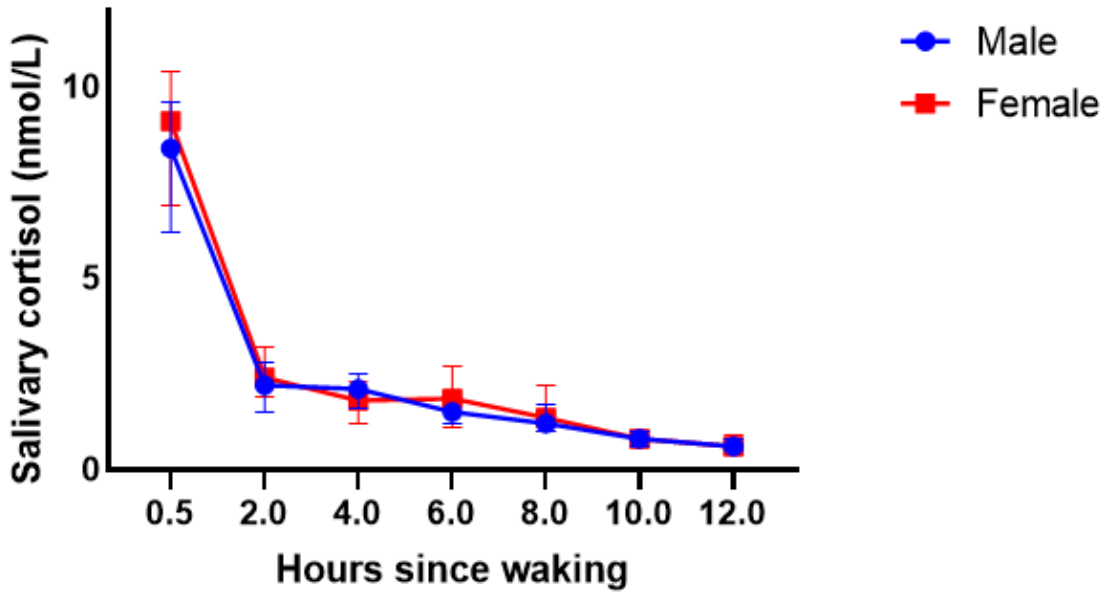


Figure 3-2:graph showing median and 95% confidence intervals of salivary cortisol (nmol/L) throughout the day and the difference between sex

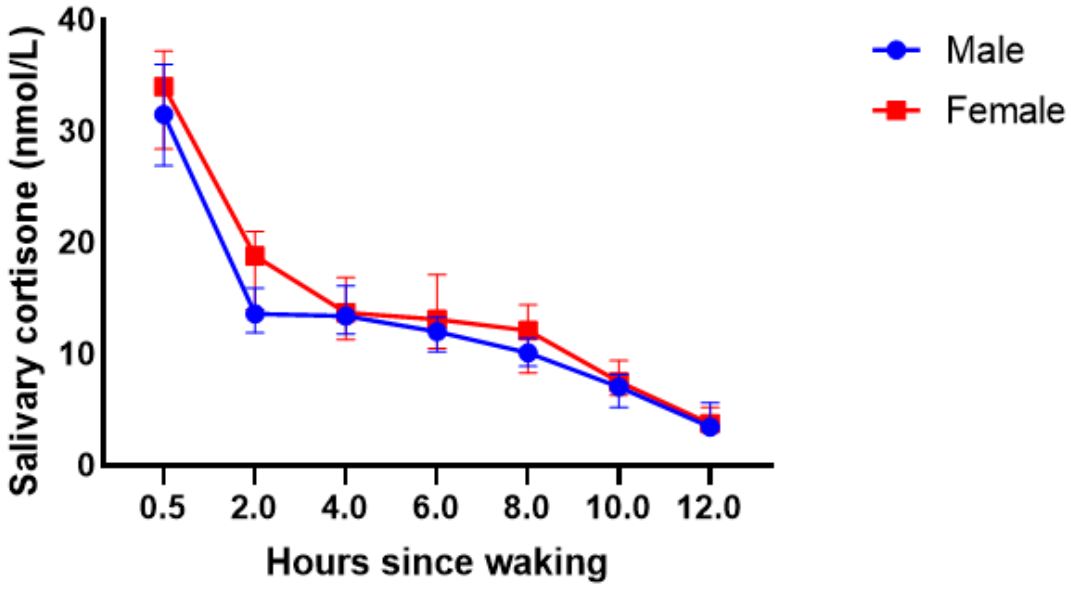


Figure 3-3: graph showing median and 95% confidence intervals of salivary cortisone (nmol/L) throughout the day and the difference between sex

3.3.2. Other salivary adrenal biomarkers

The androgens testosterone, A4, 11KT and 11OHA4 were also measured in a subgroup of 52 (30 male) of the 86 participants aged 10.4 ± 3.9 (5.0-17.5) years. Median BMI SDS was 0.3 (IQR -0.2 – 1.3) and median height SDS was 0.4 (IQR -0.3 – 1.01).

All hormones showed a circadian rhythm, with a steep decline between measurements made 30 minutes and 2 hours after waking. Cut off values of <9 years in females and <10 years in males have been used to define pubertal status. This was an estimate as tanner staging was not performed.

3.3.2.1. Classical pathway

3.3.2.1.1. Testosterone

Salivary testosterone concentrations can be seen throughout the day in Figure 3-4, concentrations in females under nine years, and males under 10 years can be seen in Figure 3-5 and the older children can be seen in Figure 3-6.

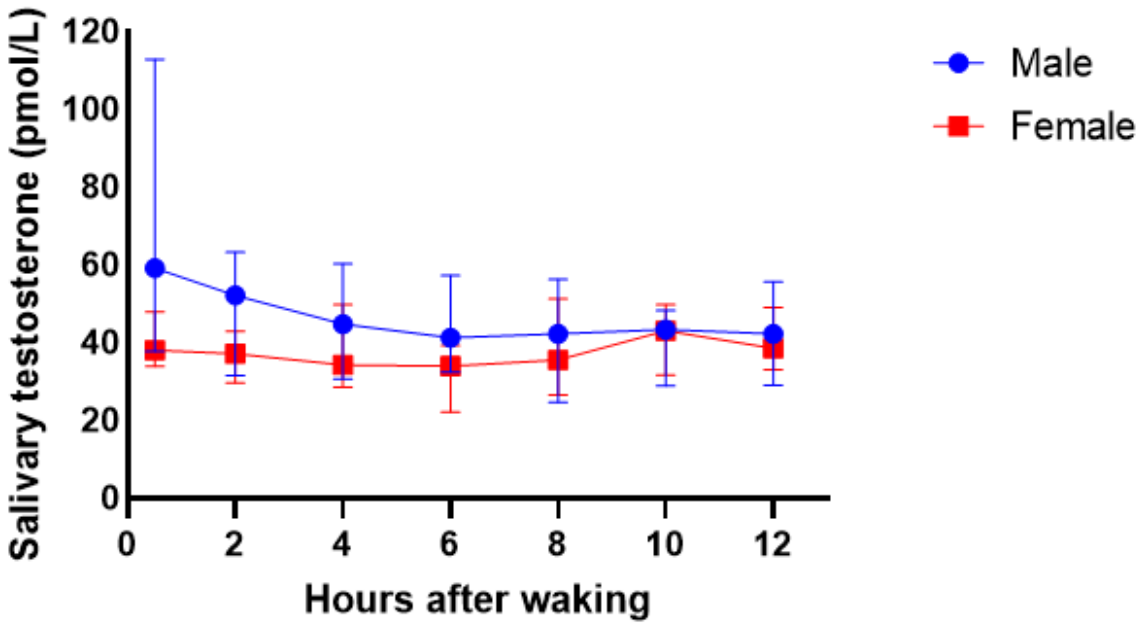


Figure 3-4: Graph showing median testosterone (pmol/L), with 95th percentiles, 30 minutes after waking, followed by 2-hourly throughout the day in males and females

Median testosterone for all males was 61.0pmol/L, and for females this was 40.0pmol/L with a p-value of <0.001.

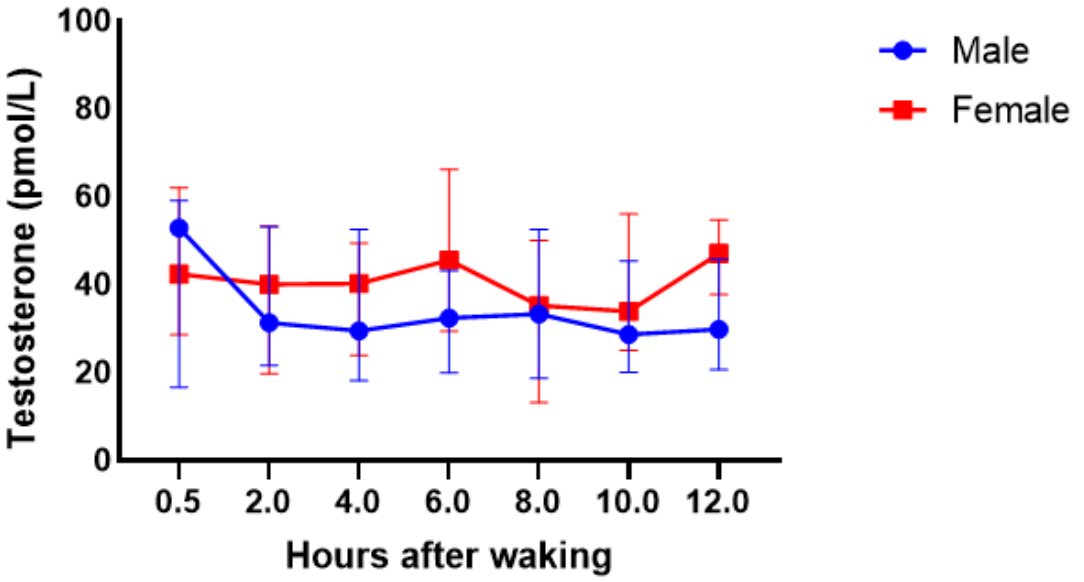


Figure 3-5: Graph showing median salivary testosterone concentrations with 5th and 95th confidence intervals in presumed pre-pubertal children

Median testosterone for presumed pre-pubertal males was 33.2pmol/L compared to females 40.6pmol/L, p value 0.13.

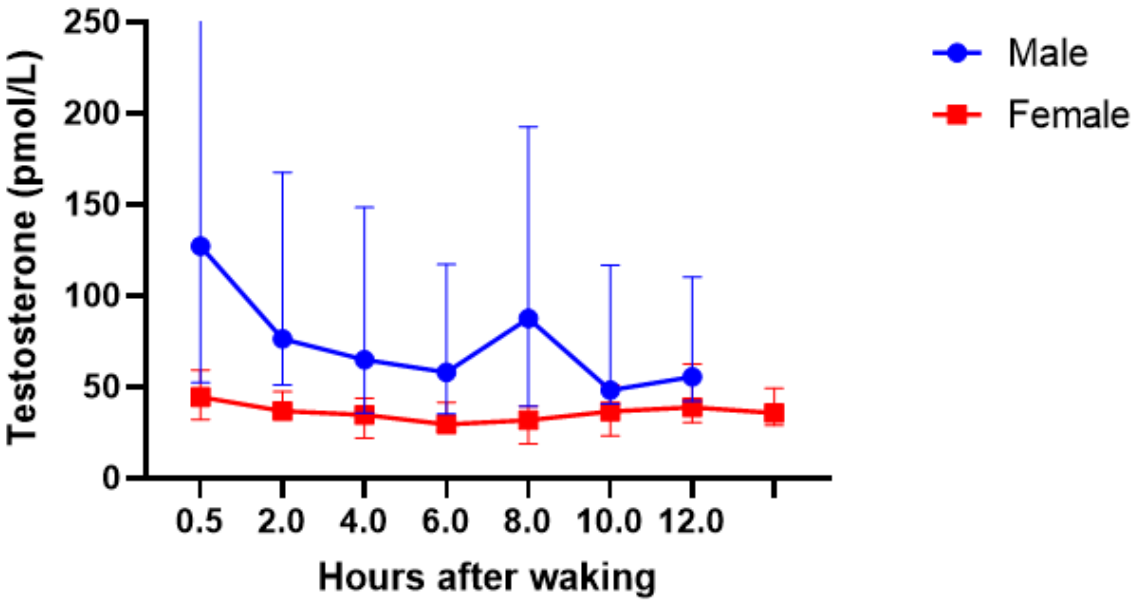


Figure 3-6: Graph showing median salivary testosterone concentrations with 5th and 95th confidence intervals in presumed post-pubertal children

Median salivary testosterone was 91.5pmol/L in presumed post-pubertal males compared to 38.4 pmol/L in presumed post pubertal females, p<0.001.

Table 3.4 shows median and 5^{th} – 95^{th} percentiles for sex and age for salivary testosterone concentrations taken throughout the day. Testosterone is higher 30 minutes after waking and then becomes steady throughout the day. Early morning salivary testosterone is higher in boys ≥ 10 years old compared to boys <10 years, p=0.004, as is the mean throughout the day (p=0.03), and the sum of all values throughout the day p=0.03.

Table 3.4: Table showing testosterone concentrations in healthy children by sex and age throughout the day, in median and 5th-95th centiles

Time after waking	Female (all, N=22), median (5^{th} – 95^{th} centile)	Female <9 years (N=12), median (5^{th} – 95^{th} centile)	Female ≥9 years (N=12), median (5^{th} – 95^{th} centile)	Male (all, N=30), years, median (5^{th} – 95^{th} centile)	Male<10 years (N=13), median (5^{th} – 95^{th} centile)	Male ≥10 years (N=16), median (5^{th} – 95^{th} centile)
30 minutes	42.6 (27.0 – 65.6)	42.4 (26.6 – 64.0)	44.4 (29.6 – 64.8)	59.1 (160 – 299.7)	52.9 (15.6 – 64.9)	127.2 (37.4 – 329.4)
2 hours	38.0 (18.3 – 61.70	40.0 (18.3 – 57.7)	36.8 (25.6 – 57.1)	52.1 (16.3 – 173.1)	31.3 (13.5 – 58.7)	76.2 (34.1 – 177.0)
4 hours	37.1 (16.2 – 49.4)	40.2 (18.6 – 49.9)	34.6 (19.0 – 44.5)	44.8 (15.7 – 165.4)	29.4 (12.6 – 55.9)	64.9 (30.7 – 224.3)
6 hours	34.2 (25.8 – 65.9)	45.5 (27.6 – 90.0)	29.5 (25.6 – 47.4)	41.2 (15.7 – 165.4)	32.4 (12.5 – 50.3)	58.0 (28.8 – 196.0)
8 hours	34.0 (13.3 – 55.1)	35.2 (12.8 – 52.8)	31.8 (18.0 – 47.3)	42.3 (14.5 – 227.0)	33.3 (13.1 – 48.5)	87.6 (26.6 – 215.2)
10 hours	35.5 (19.1 – 59.6)	33.9 (15.9 – 57.9)	36.5 (21.0 – 83.0)	43.4 (15.7 – 140.1)	28.6 (13.3 – 55.6)	48.2 (30.1 – 170.7)
12 hours	43.0 (28.9 – 62.8)	47.0 (38.3 – 53.7)	38.9 (27.2 – 63.1)	42.2 (18.3 – 110.0)	29.7 (22.5 – 64.4)	55.6 (I41.9 – 134.0)
Median of Means	**38.5 (28.5 – 49.9)**	**41.5 (26.6 – 50.2)**	**35.8 (29.0 – 49.60)**	**44.1 (24.6 – 179.6)**	**33.9 (22.5 – 64.4)**	**83.9 (35.8 – 206.3)**

N: number.

3.3.2.2. Androstenedione

Salivary A4 in all children, those presumed to be pre-pubertal and those who are presumed to be post pubertal can be seen in Figure 3-7, Figure 3-8 and Figure 3-9 respectively. Median salivary A4 in all males was 120.6pmol/L, compared to 155.0 pmol/L in females, p=0.004. The median for pre-pubertal males was 135.2pmol/L compared to 150.8pmol/L in females, p- = 0.09. Presumed post pubertal males had a median salivary A4 if 106.3pmol/L compared to 159.6pmol/L in females, p -0.004. Age and sex should be considered when interpreting normal A4 concentrations in saliva. Serum concentrations of A4 have been shown to be higher in females compared to males previously, particularly in peri-puberty and post puberty [149, 150]. These findings are reflected in salivary concentrations of A4.

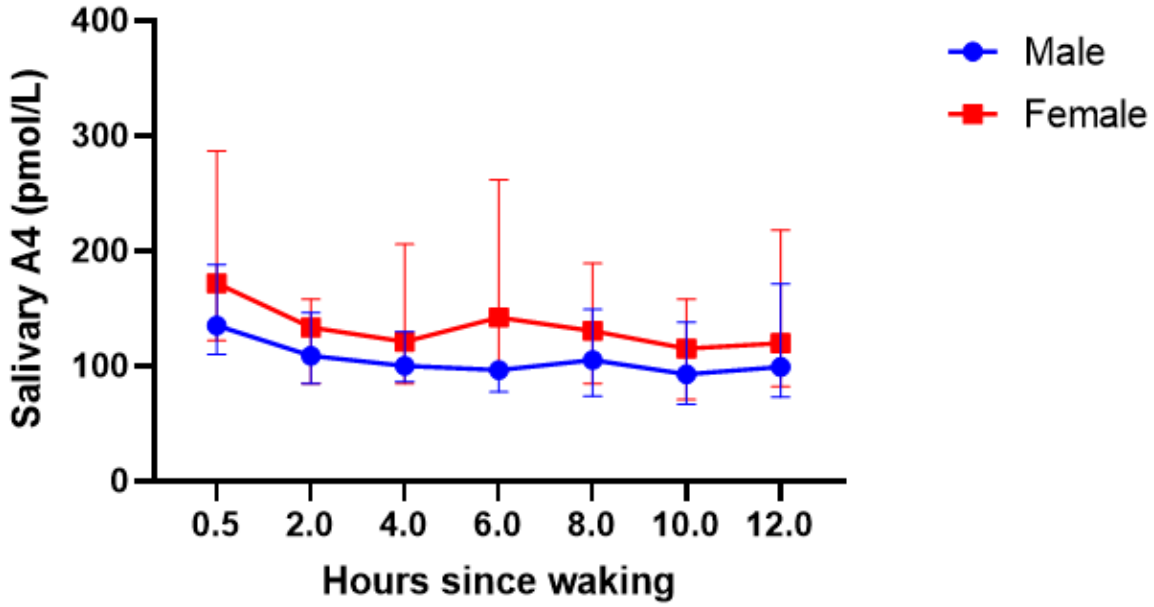


Figure 3-7: Graph showing median salivary A4 concentrations, with 5th and 95th centiles, 30 minutes after waking and two-hourly throughout the day in boys and girls

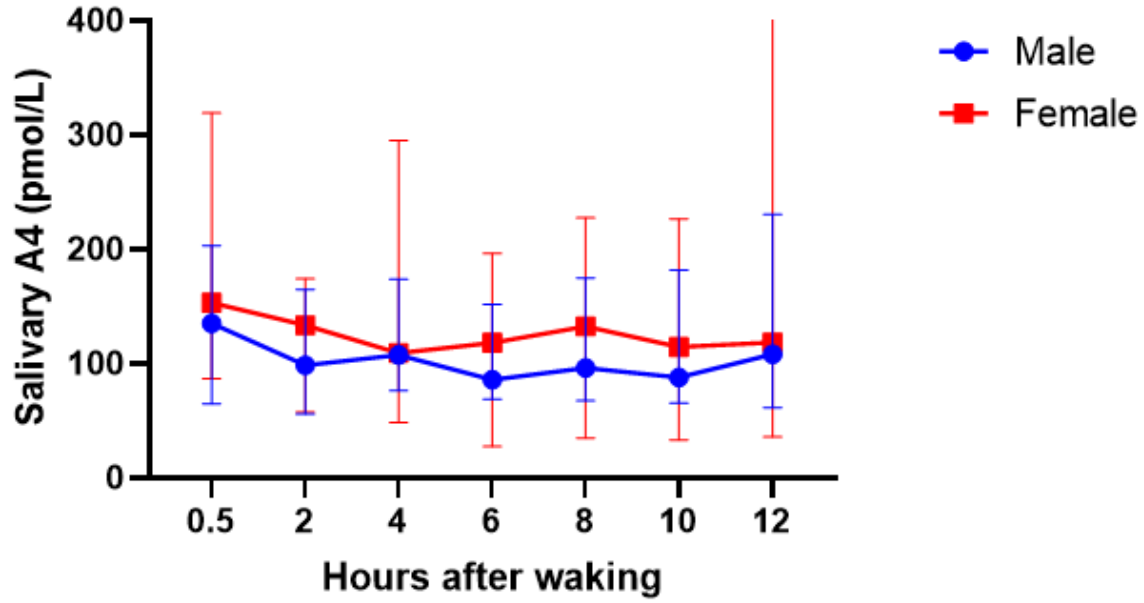


Figure 3-8: Graph showing median salivary A4 concentrations (pmol/L), with 5th and 95th percentiles throughout the day in pre-pubertal male and female children throughout the day

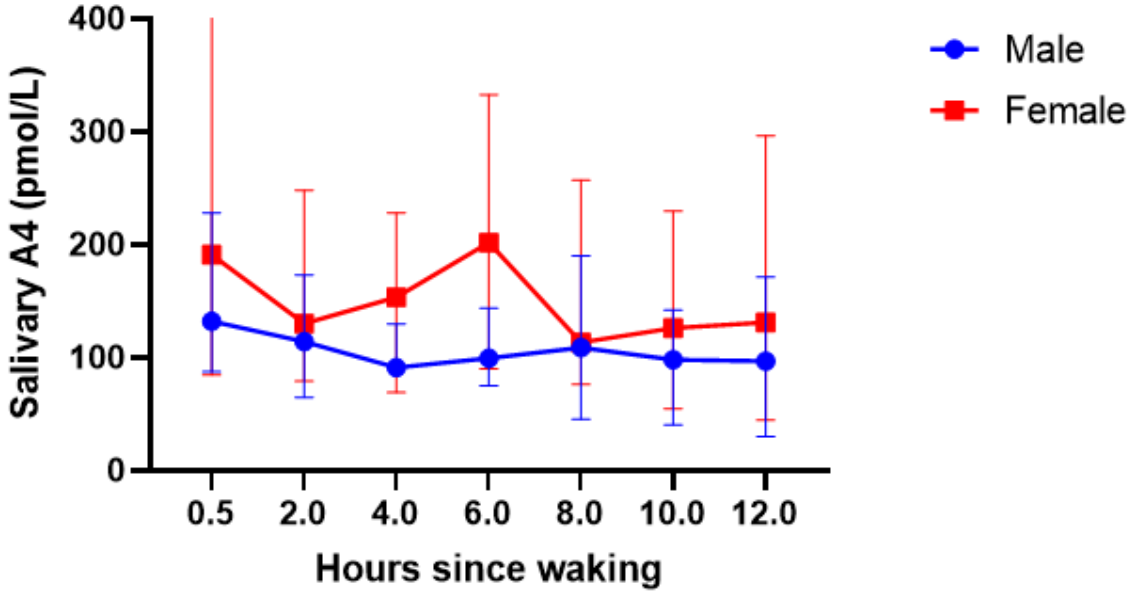


Figure 3-9: Graph showing median salivary A4 concentrations (pmol/L), with 5th and 95th percentiles throughout the day in post-pubertal male and female children throughout the day

Table 3.5 shows median, 5th and 95th percentile concentrations of A4 in female and male healthy children. No pre and post pubertal changes can be seen (Figure 3-10) with a median pre-pubertal concentration of 142.5pmol/L and post-pubertal concentration of 138.2pmol/L, p=0.54.

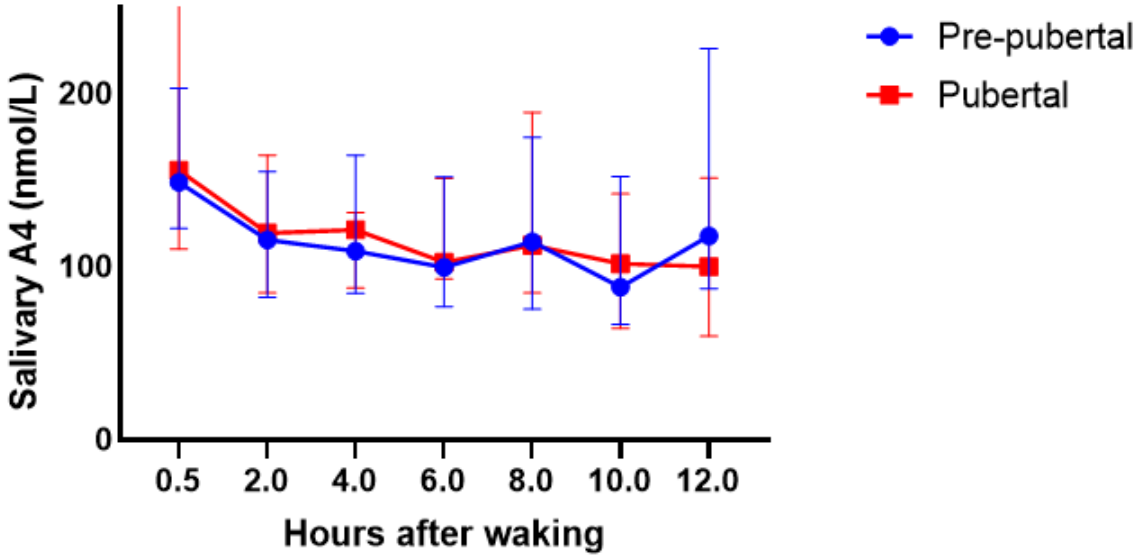


Figure 3-10: Graph showing median salivary A4 concentrations with 5th and 95th centiles in pre-pubertal compared to post pubertal children

Table 3.5: Table showing median, 5th and 95th percentile A4 concentrations in a cohort of healthy children aged 5-18 years

Time after waking	Female (all, N=23), median (5^{th} – 95^{th} centile)	Female <9 years (N=12), median (5^{th} – 95^{th} centile)	Female ≥9 years (N=11), median (5^{th} – 95^{th} centile)	Male (all, N=29), years, median (5^{th} – 95^{th} centile)	Male<10 years (n=13), median (5^{th} – 95^{th} centile)	Male ≥10 years (N=16), median (5^{th} – 95^{th} centile)
30 minutes	171.7 (55.6 – 508.2)	153.2 (63.1 – 491.8)	191.1 (69.0 – 475.3)	135.0 (47.1 – 315.7)	135.0 (34.2 – 282.9)	132.0 (72.4 – 305.0)
2 hours	133.5 (57.6 – 358.7)	133.5 (38.2 – 326.6)	130.3 (74.1 – 308.9)	108.8 (39.1 – 240.6)	98.6 (39.4 – 250.7)	114.2 (38.3 – 221.5)
4 hours	121.3 (35.5 – 358.7)	109.0 (31.0 – 400.2)	153.6 (50.6 – 229.3)	100.0 (45.2 – 259.4)	107.4 (48.2 – 296.3)	91.2 (43.2 – 161.0)
6 hours	142.1 (27.5 – 332.7)	118.1 (20.7 – 234.9)	201.9 (84.8 – 364.8)	96.7 (42.3 – 257.8)	85.8 (49.8 – 295.2)	99.5 (33.7 – 175.5)
8 hours	130.5 (30.1 – 352.3)	132.6 (28.6 – 289.3)	113.4 (48.8 – 328.4)	105.1 (32.7 – 307.0)	96.1 (33.2 – 393.7)	108.7 (35.0 – 280.3)
10 hours	115.0 (32.0 – 323.5)	114.4 (22.8 – 331.8)	126.0 (51.4 – 282.3)	92.9 (28.7 – 296.5)	87.8 (40.8 – 321.0)	98.1 (20.4 – 222.6)
12 hours	119.7 (41.9 – 345.4)	118.5 (60.9 – 381.7)	131.0 (49.1 – 268.6)	99.1 (30.2 – 266.2)	108.2 (44.6 – 276.4)	96.8 (19.3 – 224.8)
Median of means	**139.1 (51.2 – 339.8)**	**136.8 (40.6 – 331.2)**	**147.8 (72.6 – 297.6)**	**107.2 (47.5 – 249.7)**	**104.8 (51.7 – 273.3)**	**112.3 (44.9 – 205.8)**

N: number.

3.3.3. 11-oxysteroid pathway metabolites

3.3.3.1. 11-KT

11KT samples were unable to be analysed or had missing data on 28 occasions (four at 30 minutes post waking, one at two hours, two at four hours, one at six hours, one at eight hours, four at ten hours and fifteen samples at 12 hours).

11KT was undetectable in 19 further samples (one sample at 2 hours (female), 3 samples at 6 hours (2 male), 2 samples at 8 hours (both male), 5 samples at 10 hours (2 male) and 8 samples at 12 hours (6 male)). Undetectable samples (<6 pmol/L) were recorded as 5 pmol/L for analysis. 11KT median, 5th and 95th centiles can be seen in Table 3.6. In males, 11KT correlates with age r=0.79 (95CI 0.58 – 0.9), p<0.0001. This is also true in girls (r=0.63, p<0.001).

Salivary 11KT concentrations throughout the day can be seen in Figure 3-11. Median salivary 11KT in males are 60.8pmol/L compared to 76.7pmol/L in females, p=0.07.

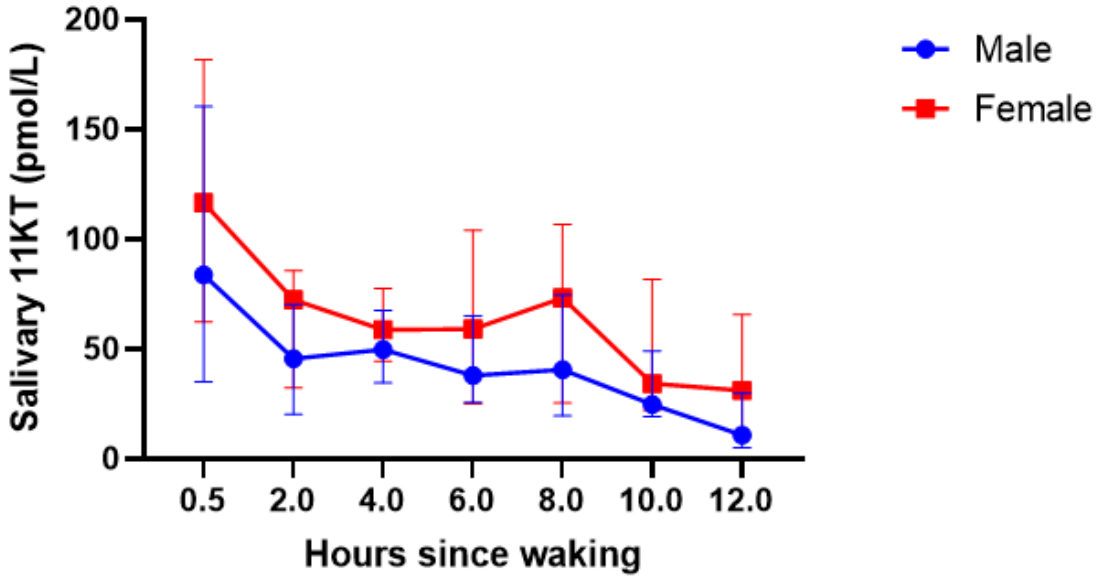


Figure 3-11: Graph showing median salivary 11KT concentrations, with 5th and 95th centiles, 30 minutes after waking and two-hourly throughout the day in boys and girls

Salivary 11KT concentrations in presumed pre-pubertal males and females and presumed post-pubertal males and females can be seen in Figure 3-12 and Figure 3-13 respectively. Median salivary 11KT was 60.8pmol/L in males <10 and females <9 years was 29.7pmol/L and

41.1 pmol/L respectively, p=0.26, whilst in the older males the median salivary 11KT concentration was 109.9pmol/L compared to 110.1pmol/L in females, p=0.8.

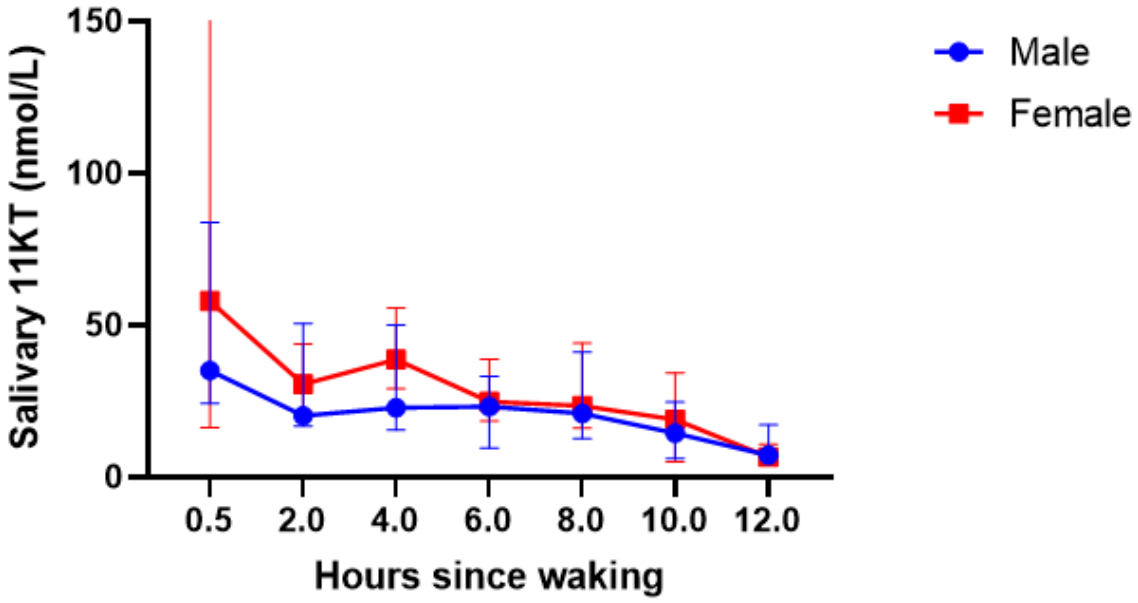


Figure 3-12: Graph showing median salivary 11KT concentrations, with 5th and 95th centiles, 30 minutes after waking and two-hourly throughout the day in presumed pre-pubertal boys and girls

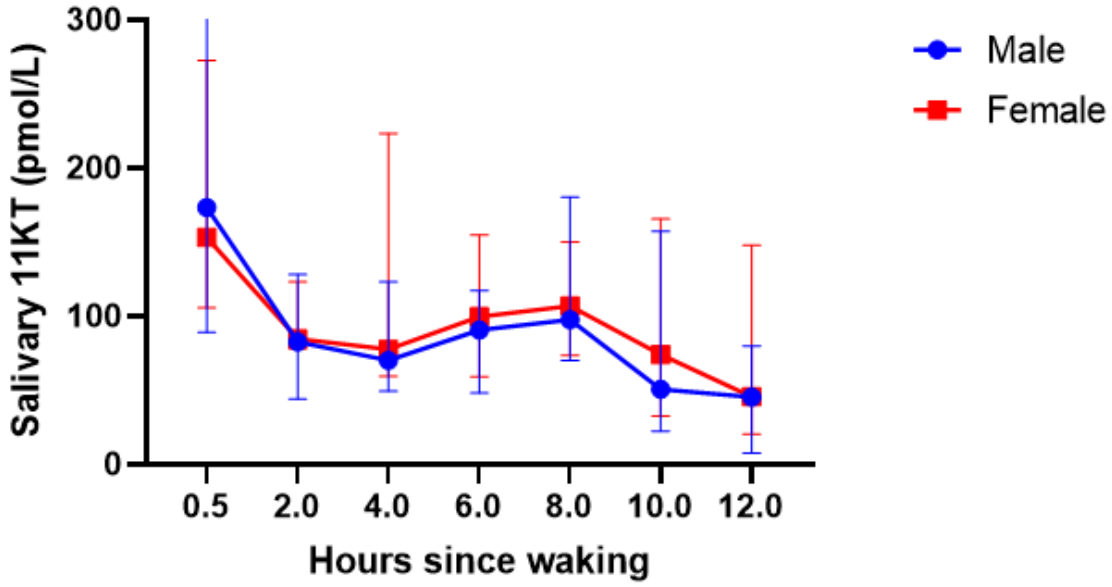


Figure 3-13: Graph showing median salivary 11KT concentrations, with 5th and 95th centiles, 30 minutes after waking and two-hourly throughout the day in presumed post-pubertal boys and girls

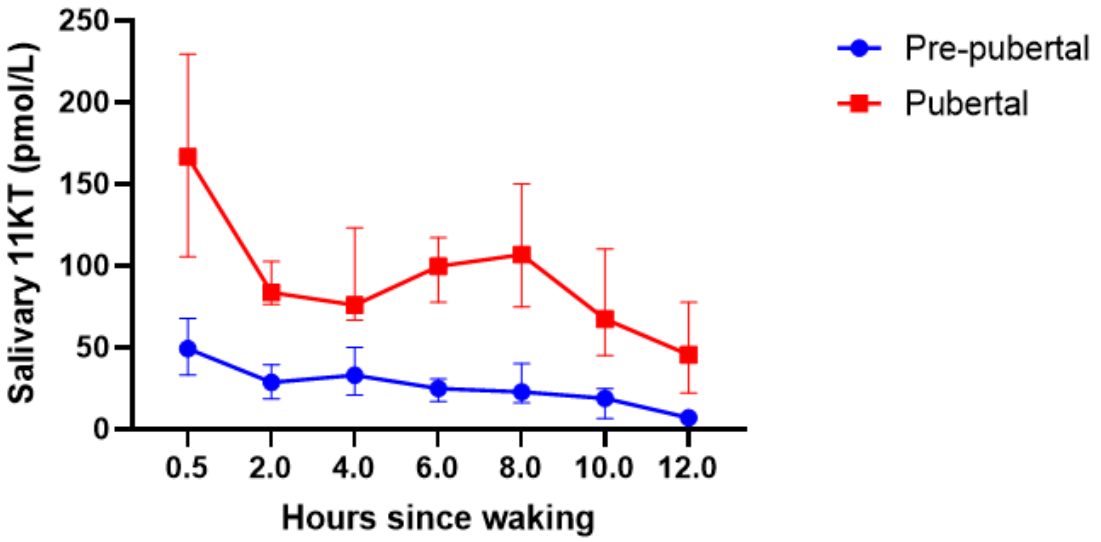


Figure 3-14: Graph showing median salivary 11KT concentrations, with 5th and 95th centiles, 30 minutes after waking and two-hourly throughout the day in both sexes' pre-puberty compared to post puberty

Salivary 11KT concentrations increase post puberty with median salivary 11KT of 35.0pmol/L in the pre-pubertal cohort and 110.0pmol/L in the post pubertal cohort, $p<0.001$ (Figure 3-14). Salivary concentrations throughout the day can be seen in Table 3.6.

Table 3.6: Table showing median, 5th and 95th centiles salivary 11-ketotestosterone concentrations in a cohort of healthy children

Time after waking	Female (all, N=23), median (5^{th} – 95^{th} centile)	Female <9 years (N=12), median (5^{th} – 95^{th} centile)	Female ≥9 years (N=11), median (5^{th} – 95^{th} centile)	Male (all, N=29), years, median (5^{th} – 95^{th} centile)	Male<10 years (n=13), median (5^{th} – 95^{th} centile)	Male ≥10 years (N=16), median (5^{th} – 95^{th} centile)
30 minutes	116.7 (19.4 – 282.8)	57.9 (16.2 – 173.0)	153.0 (74.3 – 296.2)	83.8 (24.0 – 349.2)	35.1 (22.4 – 104.2)	173.3 (59.5 – 362.7)
2 hours	72.4 (13.3 – 170.1)	30.5 (8.8 – 109.2)	84.7 (71.5 – 212.0)	45.6 (9.3 – 143.6)	20.2 (6.9 – 62.8)	82.5 (22.5 – 180.2)
4 hours	58.7 (16.3 – 229.1)	38.7 (11.7 – 69.3)	77.6 (49.7 – 278.2)	49.7 (14.9 – 202.0)	22.8 (14.0 – 80.2)	70.2 (44.6 – 346.4)
6 hours	59.0 (14.1 – 219.7)	24.8 (11.3 – 177.3)	99.6 (26.0 – 206.8)	37.8 (6.3 – 151.9)	23.2 (<6.0 – 51.8)	90.7 (40.9 – 259.8)
8 hours	73.3 (14.3 – 158.1)	23.4 (13.3 – 118.0)	106.8 (30.6 – 186.1)	40.5 (<6.0 – 177.8)	20.9 (<6.0 – 68.5)	97.7 (42.8 – 255.8)
10 hours	34.3 (<6.0 – 206.4)	18.9 (<6.0 – 89.3)	74.2 (30.4 – 224.7)	24.7 (<6.0 – 161.6)	14.4 (<6.0 – 39.9)	50.7 (14.2 – 198.7)
12 hours	31.0 (<6.0 – 217.4)	6.6 (<6.0 – 10.7)	45.5 (14.8 – 244.6)	10.7 (<6.0 – 77.50	7.2 (<6.0 – 19.1)	45.3 (9.3 – 79.0)
Median of means	**70.3 (14.8 – 176.6)**	**34.8 (10.8 – 104.2)**	**100.4 (57.0 – 220.0)**	**45.4 (11.3 – 204.4)**	**22.9 (9.7 – 55.8)**	**96.3 (37.5 – 255.8)**

N: number.

3.3.3.2. 11OHA4

Salivary concentrations of 11OHA4 were undetectable <45pmol/L. Salivary 11OHA4 concentrations between males and females can be seen in Figure 3-15 with a median concentration of 86.3pmol/L in males and 75.6pmol/L in females, p=0.9. As continuous variables 11OHA4 does not correlate with age in boys (r= -0.17, p=0.38) or in girls (r =-0.11, p 0.63). Differences when using categorical cut offs for presumed pubertal status can be seen in Figure 3-16 and Figure 3-17with median concentration of 86.3pmol/L in presumed pre-pubertal males of 103.4pmol/L compared to 109.0 in females, p=0.7. Post pubertal males have a median of 62.3pmol/L compared to 61.3pmol/L in females, p=0.7.

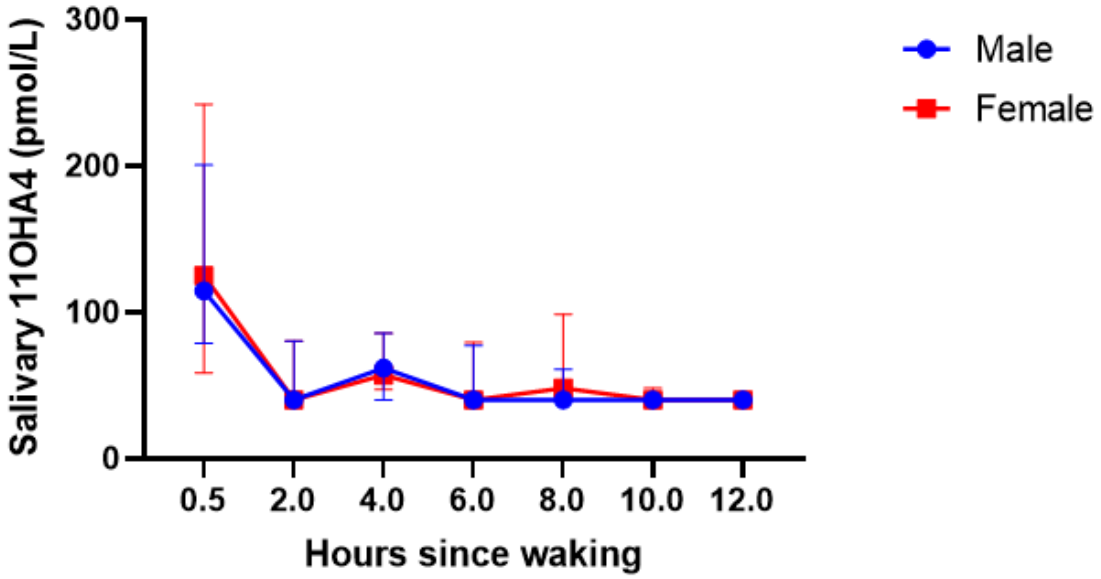


Figure 3-15: Graph showing median 11B-hydroxyandrostenedione concentrations, with 5th and 95th centiles, in males and females 30 minutes after waking and two-hourly thereafter

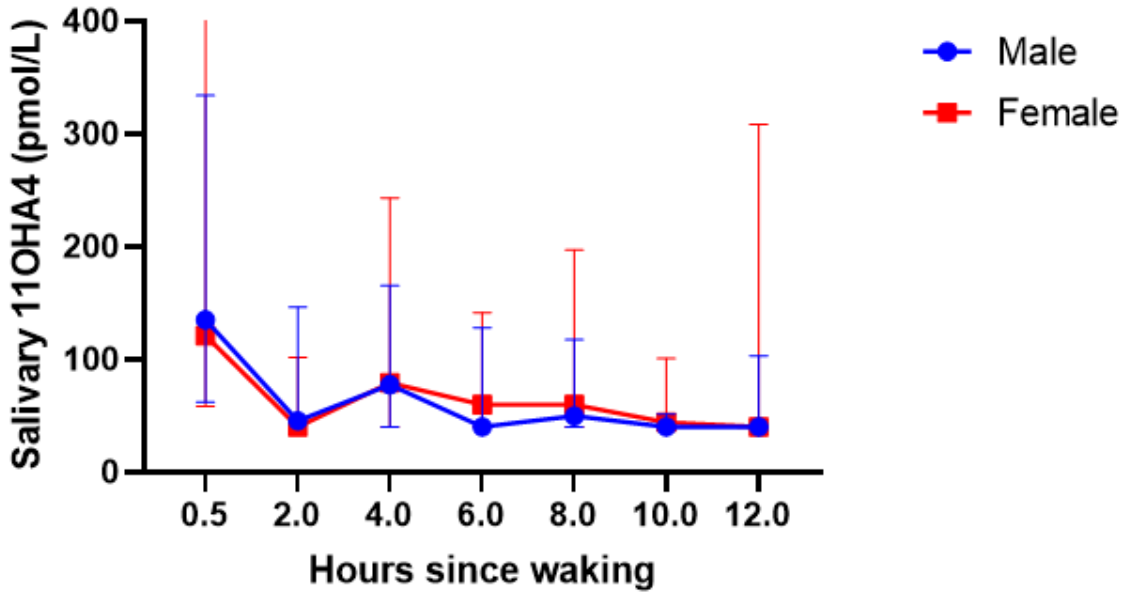


Figure 3-16: Graph showing median 11B-hydroxyandrostenedione concentrations, with 5th and 95th centiles, in presumed pre-pubertal males and females 30 minutes after waking and two-hourly thereafter

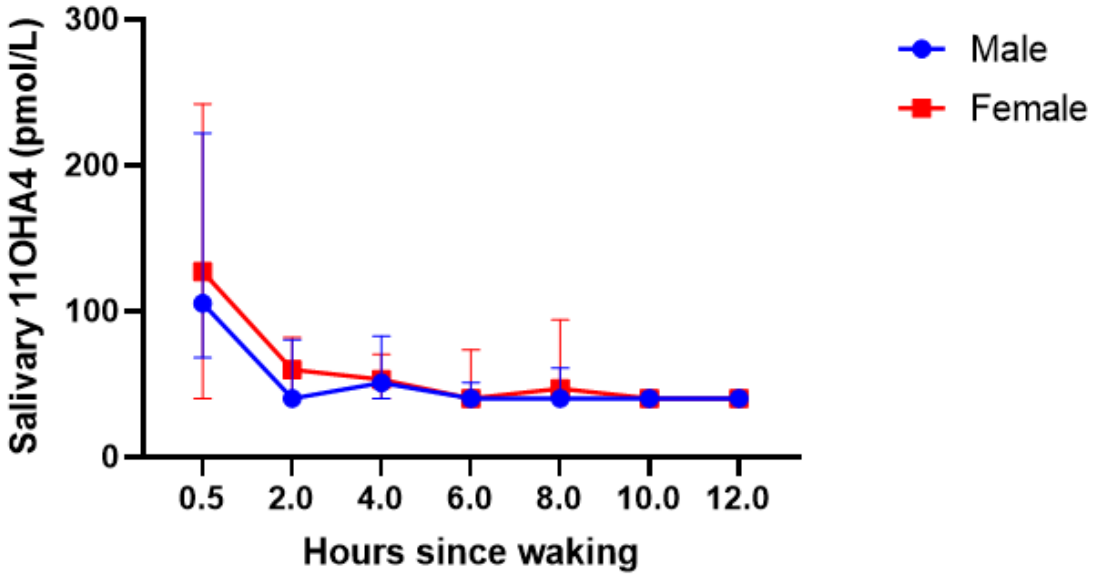


Figure 3-17: Graph showing median 11B-hydroxyandrostenedione concentrations, with 5th and 95th centiles, in presumed post-pubertal males and females 30 minutes after waking and two-hourly thereafter

Pre and post pubertal changes are seen in salivary 11OHA4 between pre-pubertal and post-pubertal children (Figure 3-18) with a median salivary concentration of 102.1pmol/L in pre-pubertal children and 58.3pmol/L in post-pubertal children, p=0.017.

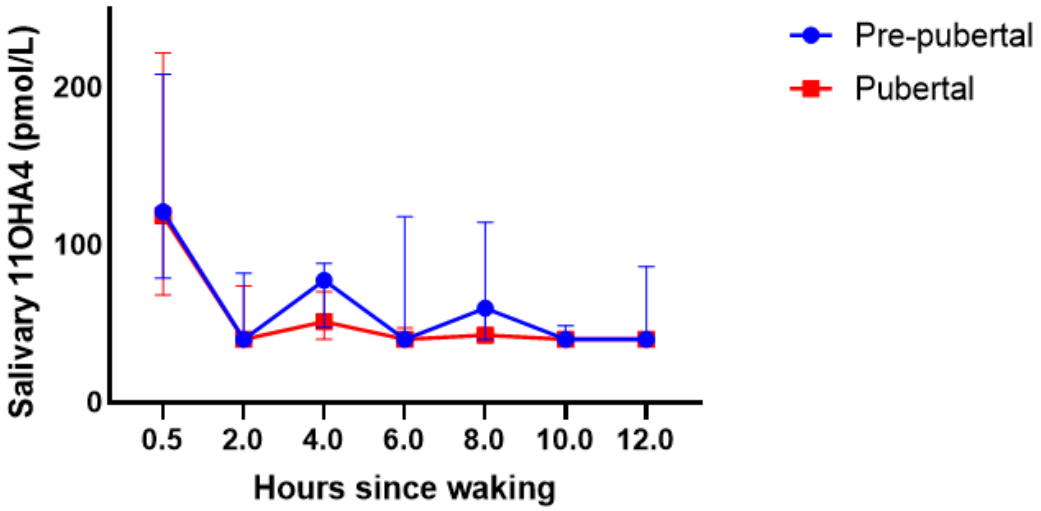


Figure 3-18:Graph showing median salivary 11OHA4 concentrations, with 5th and 95th centiles, 30 minutes after waking and two-hourly throughout the day in both sexes' pre-puberty compared to post puberty

Table 3.7: Table showing median and 5th-95th percentiles for salivary 11OHA4 concentrations in healthy children

Time after waking	Female (all, N=23), median (5th – 95th centile)	Female <9 years (N=12), median (5th – 95th centile)	Female ≥9 years (N=11), median (5th – 95th centile)	Male (all, N=29), years, median (5th – 95th centile)	Male<10 years (n=13), median (5th – 95th centile)	Male ≥10 years (N=16), median (5th – 95th centile)
30 minutes	125.1 (<45.0 – 480.2)	121.1 (47.9 – 547.5)	127.2 (<45.0 – 353.6)	114.6 (47.2 – 516.9)	135.0 (48.6 – 575.0)	105.4 (34.0 – 367.3)
2 hours	<45.0 (<45.0 – 157.6)	<45.0 (<45.0 – 243.7)	59.8 (<45.0 – 96.8)	<45.0 (<45.0 – 270.8)	45.6 (<45.0 – 340.4)	<45.0 (<45.0 – 88.0)
4 hours	57.1 (<45.0 – 421.3)	79.0 (<45.0 -488.4)	53.1 (<45.0 – 88.7)	61.9 (<45.0 – 221.9)	77.6 (<45.0 – 284.4)	50.9 (<45.0 – 135.0)
6 hours	<45.0 (<45.0 – 192.1)	59.7 (<45.0 – 213.8)	<45.0 (<45.0 – 150.5)	<45.0 (<45 – 214.0)	<45.0 (<45.0 – 254.7)	<45.0 (<45.0 – 120.8)
8 hours	48.0 (<45.0 – 194.6)	59.9 (<45.0 – 251.5)	46.9 (<45.0 – 115.7)	<45.0 (<45.0 – 189.7)	50.0 (<45.0 – 291.6)	<45.0 (<45.0 – 168.2)
10 hours	<45.0 (<45.0 – 131.6)	<45.0 (<45.0 – 225.9)	<45.0 (<45.0 – <45.0)	<45.0 (<45.0 – 235.7)	<45.0 (<45.0 – 253.8)	<45.0 (<45.0 – 165.2)
12 hours	<45.0 (<45.0 – 326.1)	<45.0 (<45.0 – 282.6)	<45.0 (<45.0 – <45.0)	<45.0 (<45.0 – 103.1)	<45.0 (<45.0 – 188.9)	<45.0 (<45.0 – 45.3)
Median of means	**69.2 (<45.0 – 226.3)**	**71.1 (<45.0 – 286.8)**	**69.2 (<45.0 – 114.6)**	**64.5 (<45.0 – 271.6)**	**68.8 (<45.0 – 333.6)**	**49.7 (<45.0 – 140.9)**

N: number.

A summary table for all salivary adrenal androgens by sex and likely pubertal status can be seen in Table 3.8.

Table 3.8: Table showing median salivary concentration for testosterone, A4, 11KT, 11B-OHA4 comparing differences between sexes and pubertal status

Hormone	**Median salivary hormone concentration and p values**						
	Prepubertal*			**Pubertal**			
	Girls, n=12	Boys, n=14	P-value**	Girls, n=12	Boys, n=16	P-value	P-value***
Testosterone (pmol/L)	40.6	33.2	0.13	38.4	91.5	<0.001	
	38.1			66.5			<0.001
A4 (pmol/L)	150.8	135.2	0.09	159.6	106.3	0.004	
	142.5			138.2			0.54
11KT (pmol/L)	41.1	29.7	0.26	110.1	109.9	0.80	
	35.0			110.0			<0.001
11OHA4 (pmol/L)	109.0	103.4	0.70	61.3	62.3	0.70	
	102.1			58.3			0.02

CI: confidence interval, n: number, pmol/L: picomoles per litre, A4: androstenedione, 11KT: 11-ketotestosterone, 11OHA4: 11-β hydroxyandrostenedione.
*pre-pubertal <9 years in girls and <10 years in boys
** boys versus girls
*** prepubertal versus pubertal and post-pubertal participants

3.4. Discussion

3.4.1. What is new?

3.4.1.1. Salivary cortisol and cortisone

Cortisol and cortisone data have been described previously [146]. This cohort is larger with a view to build on previous findings, increasing the data available to assess salivary cortisol and cortisone and formulate reference ranges, whilst also adding further assessment of other adrenal biomarkers. Salivary cortisol is often undetectable and salivary cortisone may be a more reliable measure. The ratio of salivary cortisone: cortisol increases throughout the day, suggesting that the circadian cortisol profile is regulated in part by changes in the relative activity of 11β-HSD type 1 and 2. Historically, it has been thought that adrenal secretion of cortisol determines the diurnal profile throughout the day. The change in ratio throughout the day suggest that 11β-HSD may also play a role by deactivating cortisol to cortisone peripherally reducing the amount of free cortisol later in the day. This is a novel finding that does not seem to have been described before.

3.4.1.2. Other salivary adrenal biomarkers

These novel data show a circadian profile of salivary androgens, and age and sex differences, in healthy children. Differences in salivary testosterone, A4, 11KT and 11OHA4 concentrations can be seen between sex and age using a cut off of eight years in girls and nine years in boys. As anticipated, testosterone was higher in boys than in girls, the converse was true for other androgens. This may be surprising, particularly for A4 concentrations, however, similar findings have been seen in studies that aimed to elicit normal serum reference ranges for A4 in children [149]. The concentration of salivary testosterone and A4 have been described recently [127]. Findings in this cohort are consistent with this literature. Both steroids show a diurnal pattern, peaking 30 minutes after waking and declining steeply within the following two hours. In a previous publication, concentrations of testosterone and A4 were higher in adults than in prepubertal children. The data presented here add to this observation, reporting that these differences are already present in puberty and late adolescence. It would have been ideal to undertake Tanner staging in the cohort of health children reported in this chapter. However, many children and young people find this examination uncomfortable, and we did not wish to deter children from participating in a

study of healthy children. Peri-pubertal ages need to be taken into consideration when interpreting these data as a reference range.

In the two 11oxC19 steroids that we measured, 11KT and 11OHA4, concentrations are higher in girls compared to boys, and this relationship is more pronounced for 11OHA4. 11KT increases in both girls and boys after nine and 10 years respectively, whilst concentrations of 11OHA4 remain similar in both sexes ($p = 0.48$). This is similar to findings that have been described in serum. 11KT concentrations in peripheral serum and adrenal vein sampling are similar in men and women, whilst testosterone was higher in men [131]. 11OHA4 was higher in girls than boys, $p<0.001$. It is a derivative of A4 which we have also shown to be higher in girls. This is likely to be a contributing factor to this difference.

3.4.2. Limitations

A number of samples were insufficient within this dataset. It is important to ensure adequate volumes of saliva for analysis. This can be difficult for some children. Salivettes were used in this study. Other salivary sampling methods such as passive drool may also be difficult for children. To ensure consistency salivettes will continue to be used. Sample detection limits are <6pmol/L for 11KT and <45pmol/L for 11OHA4. This seems to be more likely later in the afternoon, with boys more likely to have undetectable concentrations, particularly for 11OHA4. This occurs at expected nadirs of adrenal hormone secretion later in the day.

54 children were included in this study. After reviewing these data, there are differences in age and sex. Therefore, to provide meaningful reference ranges the study will re-open to recruitment to increase the number of healthy participants within each study group. Power calculations have determined that 134 children in total are likely to be able to provide sufficient data to give more accurate reference ranges.

3.4.3. Clinical implications for care

In the future, these data can be used as normative reference ranges. Salivary cortisone seems to be more useful than salivary cortisol, which is often undetectable, particularly later in the day.

Salivary samples can be collected at home with particular attention to obtaining samples thirty minutes after waking. Reference ranges will differ between those taken at 9am, when hospital phlebotomy services usually open, compared to those that can be taken at home,

after waking and brought to the hospital later throughout the day. A true cortisol awakening response is likely to be able to be reported. Saliva samples are thought to be stable for up to five days which may mean that it would be possible to post them and avoid a hospital attendance.

Adrenal biomarkers such as testosterone and A4 in serum are already used to guide care in CAH. These data allow salivary sampling reference ranges to be obtained with a greater understanding of how age and sex after normative concentrations. 11KT and 11OHA4 are not currently used in clinical practice. However, knowledge surrounding these biomarkers are increasing. These normative data can be used to guide future research for their clinical use. They may be able to be used to diagnoses CAH, PCOS and adrenarche. They also have the potential to guide treatment.

3.4.4. Future research

Following on from these data, further studies would be useful comparing these normative concentrations to those with CAH, PCOS and adrenarche. Chapter 4 and 5 go on to use these data for comparison for children with primary and secondary AI. Salivary cortisol and cortisone concentrations have been compared with salivary concentrations from both populations. They can be used to determine whether the child or young person is on the correct dose of hydrocortisone by comparing salivary cortisone to these normative data. Salivary cortisone follows a diurnal pattern in saliva, also. Newer hydrocortisone treatments can be compared against these data to investigate whether they show a similar diurnal profile. The other adrenal biomarkers, testosterone, A4, 11KT and 11OHA4, have been compared with salivary concentrations seen in the group with primary AI. These are just two examples of how these data are useful going forward. Future research assessing adrenal biomarkers in children and young people with adrenarche or PCOS may allow cut-offs to support or refute a diagnosis. They may also be useful for monitoring disease in CAH. Further research is required as to what concentrations are appropriate for optimal control. 17OHP is a good marker of overtreatment if it is normal or suppressed. A4 is a marker of undertreatment if it is high. 11KT or 11OHA4 may be useful to provide further information to avoid both under and over treatment.

4. Chapter 4: Glucose regulation and cardiovascular health in children and young people with primary AI (GRACE1)

4.1. Introduction

In this chapter I will discuss the study of glucose regulation and cardiovascular health in children and young people with primary AI (GRACE1). As outlined in the introduction there are data highlighting that hypoglycaemia may be of concern in children and young people with AI (see section 1.4). CGM monitoring for seven days allows trends in glucose to be assessed. Increased incidence of cardiovascular risk factors has been found in children with CAH and other forms of AI (see section 1.5.2). Children underwent assessment of their clinic BP, BP over 24-hour if ≥ 10 years old, CIMT, FMD and biochemical markers were used to assess metabolic health. Salivary biomarkers assessing cortisol, cortisone, testosterone, A4, 11KT and 11OHA4 will also highlight salivary hormone profiles throughout the day in participants.

4.2. Methodology

4.2.1. Aims and objectives

- To describe glucose, BP and salivary cortisol and cortisone profiles, and biochemical and vascular markers of vascular health to:
    - Report the prevalence, frequency and severity of hypoglycaemia in the largest cohort of children with AI studied to date, and its relationship with cortisol profiles.
    - Examine for associations between cortisol and cortisone profiles, BP disruption, markers of vascular endothelial dysfunction and biochemical determinants of cardiovascular risk to identify potential treatment targets.

4.2.2. Primary outcome

- Number of participants with glucose measurement <3mmol/L for more than 2% of the time [38].

4.2.3. Secondary outcomes

- Average systolic and diastolic clinic BP and, in those old enough, for a 24-hour period by use of ABPM [151].
- Number of participants with evidence of vascular dysfunction determined by measurement of CIMT and brachial artery flow FMD.
- Number of participants with insulin resistance defined by a rise in HOMA-IR for age and sex [152].

- AUC and mean salivary cortisol and cortisone throughout the day versus hydrocortisone dose (mg/m^2 body surface area/day).
- AUC and salivary cortisol and cortisone throughout the day versus BP, mean glucose on CGMS and HOMA-IR.
- Hydrocortisone dose versus BP and mean glucose on CGMS and HOMA-IR.
- Salivary cortisol and cortisone concentration during periods of hypoglycaemia / hypotension / hypertension.
- Concentration of plasma metanephrines versus BP in participants treated with fludrocortisone.
- Concentration of plasma leptin versus BP in participants treated with fludrocortisone.

4.2.4. Inclusion criteria

- Patients with primary AI age <18yrs.
- Written informed consent and where appropriate, assent.

4.2.5. Exclusion criteria

- Patients with additional diagnoses or treatment likely to influence blood glucose or BP.
- Patients aged <5yrs were excluded from studies of cardiovascular function.

4.2.6. Sample size

A power calculation was performed to estimate the number of patients required to identify a significant difference in the duration of hypoglycaemia in study patients compared to a cohort of healthy children [38]. If the assumption is made that the true proportion of those meeting criteria is 1% 16 participants are required to estimate proportion with 5% precision. If true proportion is 2% 31 participants are required.

4.2.7. Ethics and governance

All aspects of the study were delivered according to the standards of Good Clinical Practice, and internal research governance standards. A paediatric endocrinologist reviewed participants' data. Clinically significant results were treated according to standard practice. If hypoglycemia was found in multiple participants, the standard of care was to be updated to ensure all patients with primary AI were screened.

Ethical approval was obtained by Liverpool (central) ethics committee prior to opening of the study in October 2020 (IRAS: 283402, REC reference 20/NW/0301). A non-substantial amendment took place in December 2021 defining new age ranges for those who wore ABPM (>9 years). A further substantial amendment was made in February 2022, allowing two further saliva samples to be obtained, one prior to the first dose of hydrocortisone in the morning and one two hours following to ensure that all participants had salivary sampling from waking to going to sleep.

4.2.8. Recruitment

48 patients had primary AI and were treated at Alder Hey Children's hospital at the time of recruitment: 43 with CAH, 4 with Addison's disease and 1 with primary AI of unknown origin. All known patients with Addison's disease and primary AI of unknown origin were recruited to the study. Of the 43 patients with CAH, 12 were not eligible (two were from the Isle of Man and were unable to travel due to COVID-19 pandemic restrictions, two had non-classical CAH, six had transitioned to adult services or moved location within the last year, and two were less than two years of age). Of the 32 eligible patients, 26 enrolled and completed the study, giving a recruitment rate of 26/32 (81.3%). A minimum of seven days was given between information about the study being shared with families and obtaining consent.

4.2.9. Study visit

4.2.9.1. Procedure

Participants attended the CRF at Alder Hey Children's Hospital at 08.00, fasted. Morning medications were taken at the patient's usual time. Informed written consent, and where appropriate assent, was taken. The following data were collected:

- Age
- Sex
- Aetiology of AI and age at diagnosis
- Medication details
- Height and weight
- Medical history
- Co-morbidities
- Birth and pregnancy history
- Family history

4.2.9.2. Salivary sampling

Saliva samples were collected using age-appropriate sampling devices (*Salimetrics*, *Newmarket*) for measurement of cortisol, cortisone and other adrenal biomarkers including 17OHP, testosterone, 11KT, A4 and 11OHA4. Participants collected saliva samples every two hours during waking hours from the start of the study visit until bedtime. Saliva samples were stored in the domestic fridge in the participants' homes. Following the substantial amendment, salivary samples were requested from families for a sample prior to taking their first hydrocortisone dose in the morning and two hours following the dose.

4.2.9.3. Vascular scanning

Vascular scanning was undertaken using ultrasound techniques. This included CIMT measurements and assessment of FMD of the brachial artery (see appendix 9.3 and 9.4 for the standard operating procedures for measurement and analysis of these procedures). Physiology relating to these procedures and their associations with cardiovascular disease risk can be seen in section 1.5.1.

4.2.9.3.1. Vascular scanning analysis

4.2.9.3.1.1. CIMT

CIMT was performed (as per appendix 9.3). The CIMT measurements were performed using Q-lab software and required a 95% success rate on each measurement. Images were analysed approximately 0.5mm from the carotid bulb. If the carotid bulb was not visible in the image, it was assumed that the carotid bulb was immediately left of the image. An average was taken of three measurements on three different images for each participant.

4.2.9.3.1.2. FMD

FMD measures dilatation of the brachial artery following the cessation of ischaemia to the forearm. FMD is mediated by nitric oxide released from endothelial cells. Reduction in FMD, below 7%, has been associated with endothelial dysfunction and increased risk of cardiovascular disease [153] (See section 1.5.1). FMD was performed as per appendix 9.4.

4.2.9.3.1.2.1. FMD reproducibility

I received training on how to perform and analyse FMD measurements. Intra-operative reproducibility was tested prior to commencing the study. A co-efficient variability of less that 10 was thought to be sufficient to proceed to measurement and analysis of study participants.

Five adults were asked to fast. They attended the clinical research facility at 8.30am. The volunteer's sitting BP was taken. They then rested supine for 15 minutes. Brachial artery FMD

measurement was performed, using compression 50mmHg higher than their sitting systolic BP. An hour following the first procedure, the volunteer returned. They remained fasted. Again, they lay supine for 15 minutes before repeating the FMD measurement. Following this, the volunteer could eat and drink. All volunteers were medical staff at Alder Hey Children's hospital, aged >18 years and self-reported as fit and healthy with no medical conditions or medications that would be thought to affect the measurement of FMD. Results can be seen in Table 4.1.

Table 4.1: Table showing reproducibility of FMD measurements in healthy, adult volunteers to demonstrate appropriate intra-operative variability

Volunteer	1st FMD measurement	2nd FMD measurement	Mean FMD measurement	Standard deviation of measurement	Coefficient variability of measurement
1	6.38	6.13	6.26	0.18	2.83
2	9.19	8.9	9.05	0.21	2.27
3	3.80	3.62	3.71	0.13	3.43
4	4.89	3.82	4.36	0.76	17.37
5	12.68	11.10	11.89	1.12	9.40
		Average co-efficient variability			**7.06**

FMD: flow mediated dilatation

These studies and analysis data were reviewed by supervisors. Greater than 100 scans were preformed (including training and study participants) during this MD project.

4.2.9.4. Biochemical testing

Local anaesthetic cream was applied ahead of venepuncture. A blood sample was collected in the supine position and analysed for:

- Insulin
- Glucose*
- Lipid profile*
- Electrolytes*
- Renin activity*
- Adrenal androgen profile*
- Leptin
- Metanephrines

- Markers of endothelial damage/dysfunction (e.g. VWF)

**samples collected annually as standard of care*

An aliquot of serum was frozen and stored in the Clinical Biochemistry Laboratory at Alder Hey at -80°.

4.2.9.4.1. HOMA-IR

Several studies have reported paediatric reference ranges for HOMA-IR [152, 154-157], considering the effect of sex, age and BMI. Shashaj et al [152] reported data for 2753 Caucasians, spanning childhood (aged 2-17.8 years). This population was the mostly closely aligned to the study population. Therefore, HOMA-IR was compared with this reference data.

HOMA-IR was calculated as:

$$HOMA - IR = \frac{insulin\ \left(\frac{mU}{L}\right) x\ fasting\ glucose\ \left(\frac{mmol}{L}\right)}{22.5}$$

$$Insulin\ \left(\frac{mU}{L}\right) = \frac{Insulin\ \left(\frac{pmol}{L}\right)}{6.945}$$

4.2.9.4.2. Leptin

It is known that leptin levels can vary with age and sex [158]. For this reason, leptin was compared against normative reference data in healthy, non-obese children and adults [158]. Data are only available for those aged six years and over, therefore for children aged two to five years the percentile has been extrapolated from normative data for children aged six years.

Breakfast was provided following blood sampling and vascular scanning.

4.2.9.5. Glucose monitoring

Participants were fitted with a Dexcom G6® CGMS. The CGMS was blinded to participants with no visual, real time read out of glucose measurements. Participants removed their CGMS monitor at home or on the CRF, depending on participant preference. The data were then downloaded.

4.2.9.6. *Ambulatory BP monitoring (ABPM)*

Participants, aged ten years and over were then fitted with the Welch Allyn® ambulatory BP. For those aged between seven and ten years, a clinical decision was made regarding the appropriateness, safety and likely compliance of the participant before fitting the BP monitor. Children aged six and under were excluded from ABPM. The BP monitor was removed at home and returned, along with the saliva samples after 24 hours.

4.2.9.7. *Exercise diary*

For seven days, participants/parents completed a diary reporting illness, injury, exercise, doses and timing of hydrocortisone dosing.

4.2.10. Statistical considerations

Continuous data was subjected to the Shapiro-Wilk test to determine the nature of its distribution and expressed as mean (SD) or median (interquartile range), as appropriate. Categorical variables are reported as counts with percentages. Continuous variables are compared using independent t-tests or Mann Whitney-U test, as appropriate. Categorical data are analysed using chi-squared test or Fishers exact test as appropriate. Correlations were performed using Spearman rank correlation analysis. Prevalence of the hypoglycaemia was calculated. Linear regression models were used to assess the relation between BP values, cortisol profiles, endothelial dysfunction and biochemical determinants of cardiovascular risk. Two-tailed P values <0.05 are considered statistically significant.

4.3. Results

4.3.1. Patient Characteristics

4.3.1.1. *General characteristics*

Twenty-six (15 male) participants were recruited to GRACE 1. Patient characteristics are summarised in Table 4.2. 21 participants had CAH (14 (58.8%) with salt wasting CAH, 7 (26.9%) with simple virilising CAH), 4 (15.4%) had Addison's disease, whilst 1 (3.8%) participant had primary AI of unknown aetiology. Of the participants with CAH, one had 3B-HSD deficiency, all others had 21-OH deficiency. The four participants with Addison's disease had evidence of adrenal antibodies. Nineteen participants received fludrocortisone. All received hydrocortisone therapy. Nineteen participants considered themselves white British, two children considered themselves 'Other white', one was mixed: white and black African, one participant was of Pakistani origin and one considered themselves 'other mixed background.' No pregnancy was complicated with maternal pre-eclampsia, hypertension or kidney disease.

Median (IQR) birth weight was 3.0kg (3.1 – 3.7). A summary table of all individuals and individual summaries of hydrocortisone dose, timing of dose, CGMS measurements and salivary cortisol and cortisone profiling of all participants can be found in appendix 9.5 and 9.6 respectively.

Table 4.2: Table showing participant characteristics for GRACE 1 cohort as a whole and by cause of primary AI

Parameter	Whole cohort N=26	CAH N = 21	Addison's disease N = 4	Primary AI – unknown aetiology, N= 1
(i) Age and length of diagnosis (median, range)				
Age, years	10.0 (2. – 18.0)	6.0 (2.0 – 17.0)	14.0 (12.0 – 16.0)	18.0
Age at diagnosis of PAI, months	4.8 (0.0 – 168.0)	0.5 (0.0 – 96.0)	155.5 (120.0 – 168.0)	15.0
Length of time diagnosed with PAI, years	5.0 (1.0 – 17.0)	6.0 (1.3 – 17.0)	2.0 (1.0 – 2.0)	3.0
(ii) Auxology (median, range)				
Height SDS	-0.1 (-2.0 – 2.6)	-0.4 (-2.0 – 2.6)	0.9 (-0.7 – 2.2)	0.52
Weight SDS	0.2 (-1.3 – 8.7)	0.0 (-1.3 – 8.7)	2.1 (1.0 – 4.7)	-0.44
BMI SDS	0.7 (-1.6 – 3.7)	0.6 (-0.7 – 3.7)	1.7 (1.0 – 2.5)	-1.6
(iii) Medication doses (median, range)				
Fludrocortisone dose, mcg	150.0 (0.0 – 200.0)	100.0 (0.0 – 175.0)	200.0 (150.0 – 200.0)	150.0
Fludrocortisone, mcg/ m^2/day	103.4 (15.6 – 269.2)	153.8 (15.6 – 269.2)	101.3 (90.9 – 107.1)	88.2
Hydrocortisone dose, mg/ m^2/day	9.9 (5.6 – 25.0)	9.4 (5.6 – 15.7)	18.4 (10.3 – 25.0)	10.3
Sick day hydrocortisone dose (mg/ m^2/day)	19.0 (12.5 – 44.3)	18.5 (12.5 – 44.3)	27.8 (20.0 – 36.4)	23.5
(iv) Cardiovascular risk factors (N, %)				
1st degree relative with cardiovascular disease	1 (3.8%)	1 (4.8%)	0 (0%)	0 (0%)
Co-existing familial hypercholesterolaemia	1 (3.8%)	1 (4.8%)	0 (0%)	0 (0%)
1st degree relative with non-familial lipid abnormalities	1 (3.8%)	1 (4.8%)	0 (0%)	0 (0%)
Parental smoking	8 (30.1%)	6 (28.6%)	2 (50%)	0 (0%)
Participant smoking	1 (3.8%)	1 (4.8%)	0 (0%)	0 (0%)

N: number, CAH: congenital adrenal hyperplasia, AI: adrenal insufficiency, SDS: standard deviation score, mcg: micrograms, mg: milligram.

4.3.1.2. *Hydrocortisone and fludrocortisone doses*

Blood pressure is thought to be affected by both hydrocortisone and fludrocortisone. Within this cohort, there was a significant difference in hydrocortisone dose between those with CAH (9.4 [8.8 – 10.1] mg/m^2/day) and Addison's Disease (18.4 [13.3 – 23.1] mg/m^2/day) using the independent samples Kruskal-Wallis test (p = 0.028) (Figure 4-1). The single patient with primary AI of unknown cause was treated with 10.3 mg/ m^2/day. Fludrocortisone doses were not significantly different between groups using the independent samples Kruskal-Wallis test (p=0.781) (Figure 4-1).

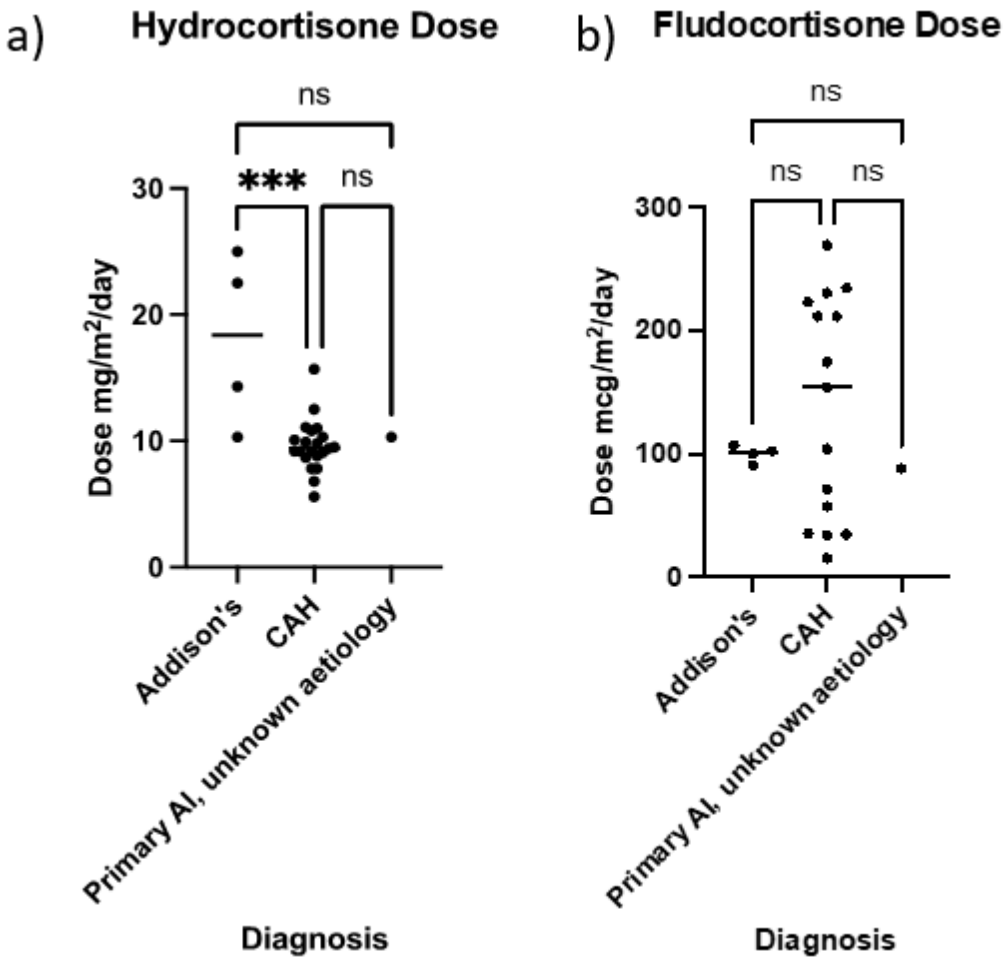


Figure 4-1: Graph showing differences in a) hydrocortisone and b) fludrocortisone doses between categories.

4.3.2. Glucose regulation

4.3.2.1. *Fasting glucose and CGM data*

Mean fasting glucose was 4.3 ± 0.5mmol/L, with the lowest glucose concentration being 3.2mmol/L. Glucose data from CGM were available in 25 / 26 participants. One child could not tolerate the monitor, and it was removed immediately after insertion. One young person

tolerated the device for two days. This young person had a history of anxiety, depression and oppositional defiant disorder. All other participants tolerated the device for 7 days. The mean, SD and percentage of time spent below 3mmol/L and above 10mmol/L are reported in Table 4.3.

Table 4.3: Mean glucose, standard deviation of measurements and period spent with hypoglycaemic and hyperglycaemic readings in study participants compared to published data from healthy controls

Parameter	GRACE1 study (n = 25)	Reference data, [38] 6 - 18 years (n = 58)
Mean glucose (1SD)	6.11 (± 0.63)	5.47 (± 0.37)
Standard deviation of measurements (1SD)	1.19 (± 0.84)	0.88 (± 0.17)
Median % of time < 3 mmol/L (IQR)	0.00 (0.00-0.20)	0.00 (0.00 – 0.20)
Median % of time > 10 mmol/L (IQR)	0.00 (0.00-0.5)	0.00 (0.00 – 0.10)

N: number, SD: standard deviation, %: percentage, IQR: interquartile range, mmol/L: millimoles per litre.

One participant had evidence of significant hyperglycaemia, particularly during sleep. She was reviewed, and a full history was taken showing no evidence of polydipsia, polyuria, lethargy or weight loss. Her pancreatic autoantibodies were measured, and were all negative. A further, unblinded, CGMS was inserted into her abdomen, on the contra-lateral side to the site of the previous CGMS. The family were also taught to check glucose measurements by finger prick measurements. There was clear discordance between the monitor and finger prick glucose measurements. Hypoglycaemia has been reported to occur when the CGMS sensor is compressed. To my knowledge hyperglycaemia has not been reported. The patient continues to monitor finger prick glucose levels intermittently, which remain within the normal range. On removing this outlier, the data are shown in Table 4.4.

Table 4.4: Table showing GRACE 1 data without outlier compared to reference population

Parameter	GRACE1 study (n = 24)	Reference data [38] 6 - 18 years (n = 58)
Mean glucose (1SD)	6.04 (± 0.53)	5.47 (± 0.37)
Standard deviation of measurements (1SD)	1.21 (± 0.86)	0.88 (± 0.17)
Median % of time < 3 mmol/L (IQR)	0.00 (0.00-0.33)	0.00 (0.00 – 0.20)
Median % of time > 10 mmol/L (IQR)	0.00 (0.00-0.43)	0.00 (0.00 – 0.10)

N: number, SD: standard deviation, %: percentage, IQR: interquartile range, mmol/L: millimoles per litre.

During the study, we noted that hypoglycaemic readings occurred with much greater frequency in the first twenty-four hours than at other times, with no clear cause (ambulatory glucose profiles can be found in appendix 9.6). This observation has been reported previously [159, 160]. For this reason, data from day 1 have been excluded, both from the GRACE 1 population and the raw data from the reference publication [49], thus capturing only data from day 2 to the removal of the sensor (see Table 4.5).

Table 4.5: Glucose parameters with and without the first 24 hours of measurements for study participants and published reference data [49]

Parameter[38]	GRACE1 study (n = 24) including measurements from the first 24 hours	Reference data including measurements from the first 24 hours [38] 6 - 18 years (n = 58)	GRACE 1 study (n = 24) excluding measurements from the first 24 hours	Reference data excluding measurements from the first 24 hours [38] 6 - 18 years (n = 58)
Mean glucose (1SD)	6.04 (± 0.53)	5.47 (± 0.37)	6.07 (± 0.58)	5.55 (± 0.36)
Standard deviation of measurements (1SD)	1.21 (± 0.86)	0.88 (± 0.17)	1.02 (± 0.55)	0.91 (± 0.17)
Median % of time < 3 mmol/L (IQR)	0.00 (0.00-0.33)	0.00 (0.00 – 0.20)	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.20)
Median % of time > 10 mmol/L (IQR)	0.00 (0.00-0.43)	0.00 (0.00 – 0.10)	0.00 (0.00 – 0.50)	0.0 0.00 – 0.10)

N: number, SD: standard deviation, %: percentage, IQR: interquartile range, mmol/L: millimoles per litre.

4.3.2.2. Comparison to primary outcome

One participant, aged four years, treated with hydrocortisone 9.2mg/m^2/day, was hypoglycaemic for 2.4% of the time, with all hypoglycaemic measurements being between midnight and midday (Figure 4-2). The dose of hydrocortisone was increased to 9.8mg/m^2/day and repeat ambulatory glucose profile showed a reduction in hypoglycaemic measurements (1.1% of time spent with blood glucose <3mmol/L) (Figure 4-3).

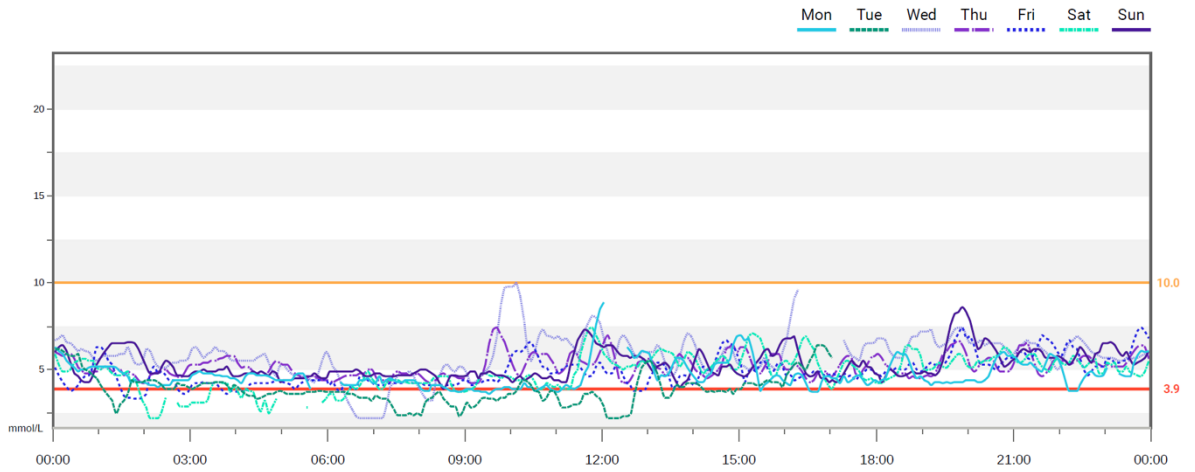


Figure 4-2: Figure showing ambulatory glucose profile for participant, aged 4 years with CAH, who was hypoglycaemic (<3mmol/L) for >2% of the time on 9.2mg/m^2/day hydrocortisone

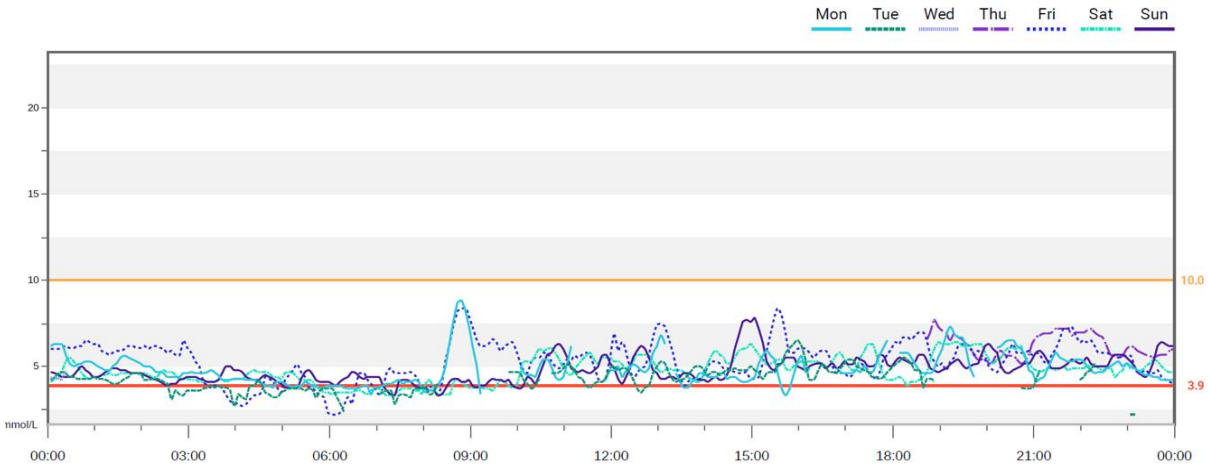


Figure 4-3: Repeat ambulatory glucose profile for participant, aged 4 years following an increase in hydrocortisone dose to 9.8mg/m^2/day

4.3.2.3. Statistical analysis

The mean glucose of the normative [38] and GRACE 1 data were normally distributed. Independent samples t-test were used to compare the means. The mean glucose concentration of healthy children and young people was 5.5 (±0.36) mmol/L. The mean glucose concentration of participants with primary AI was 6.01 (±0.55) mmol/L. Mean glucose

concentrations in mmol/L between groups can be seen in Figure 4-4. The independent t-test showed a significant difference (p<0.001), with a 95% CI that there is between a 0.2 and 0.7mmol/L difference between the two groups. The co-efficient of variation of glucose levels did not differ between two groups, p=0.15 (Figure 4-4). Mean glucose on CGMS did not correlate with hydrocortisone dose.

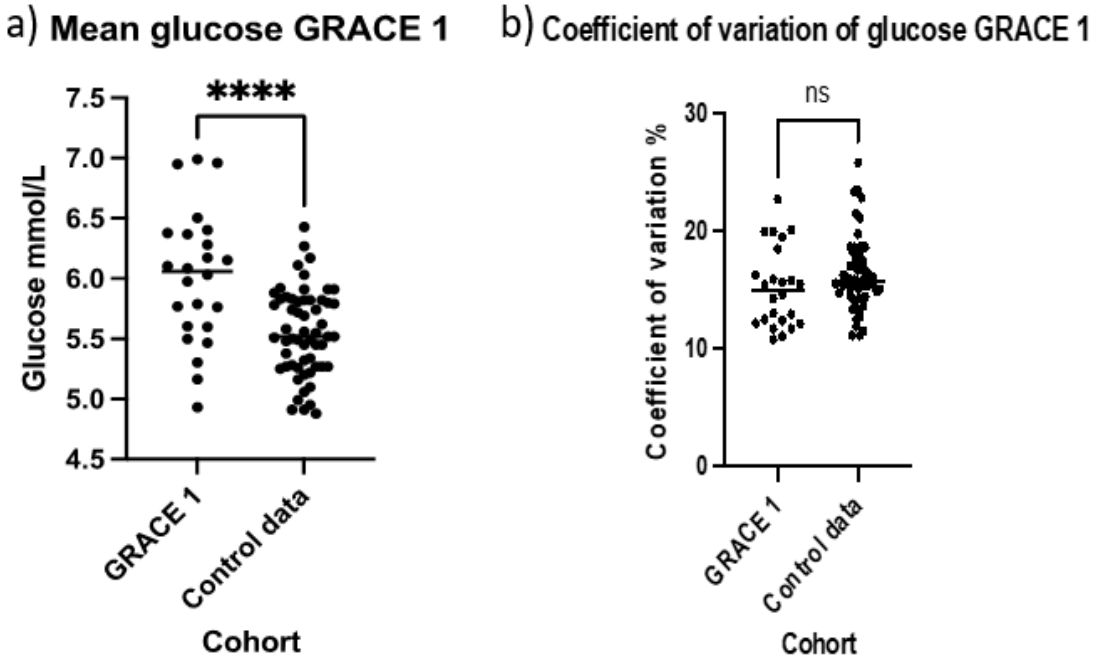


Figure 4-4: Mean glucose concentration and co-efficient of variation, over six days in patients with primary AI and published data from healthy controls

The distribution across categories (>10mmol/L, >8.89mmol/L, >7.78mmol/L, between 3.39-7.8, <3.9, <3.3 and <3 mmol/L) were not normally distributed and therefore a Mann Whitney U test was performed to compare groups. The results are shown in Table 4.6.

Table 4.6: Table showing GRACE 1 participants glucose data compared to reference data

	GRACE 1 participants	Control subjects	*P*-value	Significance after Bonferroni correction
(i) Percentage of time spent with glucose readings in each category, median (IQR)				
>10.00 mmol/L	0.0 (0.0 – 0.1)	0.0 (0.0 – 0.1)	0.60	NS
>8.89 mmol/L	0.3 (0.1 – 0.88)	0.0 (0.0 – 0.5)	0.06	NS
>7.78 mmol/L	11.9 (1.0 – 3.4)	1.1 (0.4 – 2.3)	0.02[a]	NS
3.39 – 7.78 mmol/L	96.4 (93.6 – 97.5)	96.8 (91.9 – 98.7)	0.12	NS
<3.39 mmol/L	1.1 (0.3 – 2.6)	1.4 (0.5 – 3.7)	<0.001 [a]	0.01
<3.33 mmol/L	0.1 (0.00 – 0.44)	0.3 (0.0 – 0.5)	0.07	NS
<3.00 mmol/L	0.0 (0.0 – 0.22)	0.1 (0.0 – 0.4)	0.15	NS

IQR: interquartile range, mmol/L: millimoles per litre, NS: non-significant
a – significant result

There was a statistical difference ($p<0.05$) between groups in glucose parameters >7.78mmol/L and glucose levels <3.9mmol/L. GRACE 1 participants were more likely to have a higher percentage of glucose levels >7.78mmol/L. However, following a Bonferroni correction this significance was lost. Even without a Bonferroni correction, this became less significant as the glucose concentrations increased. Healthy children were more likely to have lower glucose concentrations (<3.9mmol/L). However, there was no significant difference at glucose values <3mmol/L.

4.3.3. Cardiovascular health

4.3.3.1. General characteristics for cardiovascular studies

The characteristics of the 21 participants who were eligible for the cardiovascular studies of endothelial dysfunction, including CIMT and FMD, are given in Table 4.7. All participants with Addison's disease and primary AI of unknown aetiology are included within this table, 16 have CAH.

Table 4.7: Characteristics of participants eligible for cardiovascular studies of endothelial dysfunction (CIMT and FMD)

Parameter	Median (IQR)
(i) Age and length of diagnosis	
Age, years	12.0 (6.8 – 14.5)
Age at diagnosis of PAI, months	13.5 (0.3 – 54.0)
Length of time diagnosed with PAI, years	6.0 (4.5 – 8.0)
(ii) Auxology (median, IQR)	
Height SDS	0.5 (-0.6 – 0.7)
BMI SDS	0.9 (-0.3 – 2.1)
(iii) Medication doses (median, IQR)	
Fludrocortisone dose, mcg	125.0 (75.0 – 168.8)
Fludrocortisone, mcg/m^2/day	95.5 (52.0 – 104.9)
Hydrocortisone dose, mg/m^2/day	10.3 (8.9 – 11.5)
Sick day hydrocortisone dose, mg/m^2/day	20.0 (18.3 – 23.4)

IQR: interquartile range, PAI: primary adrenal insufficiency, SDS: standard deviation score, BMI: body mass index, mcg: micrograms, mg: milligrams.

4.3.3.2. BP

Clinic BP was measured on the CRF in 26 participants. Median percentile, IQR and range of percentiles are reported in Table 4.8.

Table 4.8: Median, interquartile range and range for systolic and diastolic clinic BP

Parameter	Median, IQR	Range
Systolic BP (percentile)	85.0 (68.0 – 95.25)	6^{th} – 99^{th}
Diastolic BP (percentile)	65.0 (41.0 – 92.3)	4^{th} – 99^{th}

IQR: interquartile range, BP: blood pressure.

In seven (26.9%) participants, systolic BP was greater than or equal to the 95^{th} percentile. In four (14.8%) participants diastolic BP was greater than the 95^{th} centile, three of whom had both a raised systolic and diastolic BP. Details of each patient are reported in Table 4.9.

Table 4.9: Table showing participant details of those with systolic and diastolic BP>95th centile for age and sex

Participant	Age (years)	Sex (M/F)	Diagnosis	BMI SDS	Fludrocortisone dose (mcg/m^2/day)	Hydrocortisone dose (mg/m^2/day)
Raised systolic and diastolic BP						
009	3	F	Salt losing CAH	-0.1	234.4	9.4
010	2	M	Salt losing CAH	0.5	223.2	9.8
024	16	M	Salt losing CAH	3.2	34.1	9.1
Raise systolic BP only						
002	4	M	Salt losing CAH	0.6	211.3	9.2
004	5	F	Salt losing CAH	0.2	57.5	9.2
006	5	F	Salt losing CAH	-0.1	269.2	10.8
021	6	M	Salt losing CAH	0.7	174.4	8.7
Raised diastolic BP only						
011	9	M	Simple virilising CAH	3.3	-	5.6
Median (IQR)	**5.0 (3.8-6.8)**			**0.55 (0.1-1.3)**	**211.3 (116.0-228.8)**	**9.2 (9.0-9.5)**

M: male, F: female, BMI: body mass index, SDS: standard deviation score, mcg: micrograms, mg: milligrams, BP: blood pressure, CAH: congenital adrenal hyperplasia, IQR: interquartile range.

Fludrocortisone dose (mcg/kg/day) is significantly correlated to BP (Figure 4-5). Using ANOVA regression modelling, it can be ascertained that fludrocortisone has a 29.6-69.5% effect on systolic BP ($p<0.0001$). Hydrocortisone dose (mg/m^2/day) did not correlate with BP.

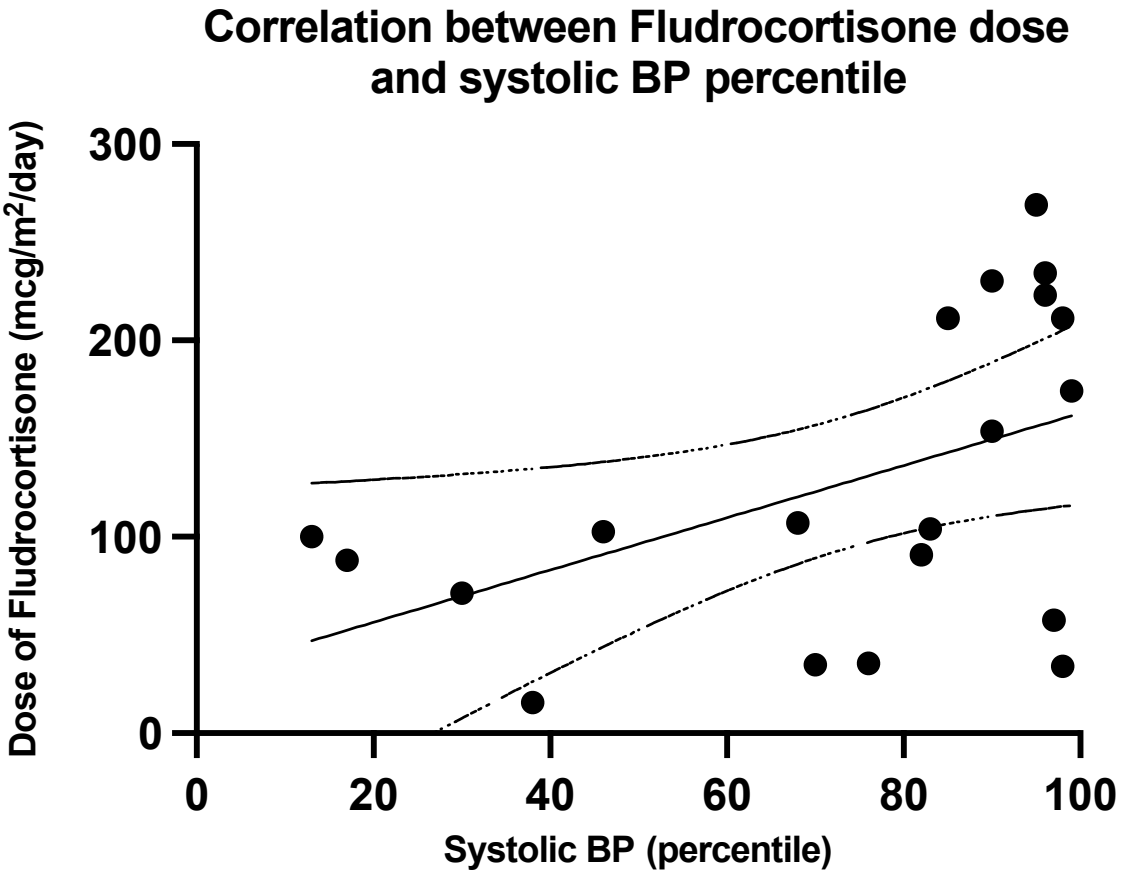


Figure 4-5:Correlation of fludrocortisone dose (mcg/m^2/day) and systolic BP percentile. r^2 0.23 (p value=0.032)

There was no correlation between plasma renin and dose of fludrocortisone (r^2, p=0.99) or between plasma renin and systolic BP percentile (r^2 0.01, p = 0.56). Systolic and diastolic BP did not differ compared to diagnosis.

4.3.3.3. Ambulatory BP monitoring (ABPM) - ≥7 years

Ambulatory BP was performed in 13 (9 males) participants, aged 14.5 (12.0 – 16.0) years. Four participants could not tolerate the BP cuff and removed it, particularly prior to bedtime. Duration of time below 5th or above 95th centile could not be ascertained as individual BP measurements were not available on reports obtained.

Table 4.10 shows data from each participant where ≥50% of readings were successful, indicating that they tolerated the BP cuff both in the day and night [161]. Three of nine (33.3%) participants showed reduced nocturnal dipping (<10% fall in their systolic BP), whilst two (22.2%) participants showed reduced nocturnal dipping in their diastolic BP (<10% fall in their diastolic BP). In one patient (11.1%), the nocturnal dip was absent in both systolic and diastolic measurements.

Table 4.10: Ambulatory BP measurements, showing systolic and diastolic BP centiles and evidence of nocturnal dipping. A fall in BP of 10% during sleeping hours defines normal 'dipping'.

Age	Sex (M/F)	BMI SDS	% successful readings	Diagnosis	Systolic BP centile	Diastolic BP centile	Systolic BP dipping (%)	Diastolic BP dipping (%)
15	F	1.9	73.7	Addison's Disease	<50th	<50th	15	22
16	F	-0.7	65.9	CAH (simple virilising)	<50th	<50th	12	24
12	M	-0.3	84.2	CAH (salt wasting)	<50th	<50th	13	23
12	M	1.4	92.5	Addison's Disease	50th-75th	50th-75th	14	15
16	M	1.0	79.6	Addison's Disease	<50th	<50th	4^{b}	15
14	F	0.7	67.7	CAH (simple virilising)	50th-75th	<50th	13	20
17	M	2.3	53.7	CAH (salt wasting)	<50th	75th-90th	7^{b}	26
13	M	2.5	90.0	Addison's Disease	90th-95$^{th\ a}$	<50th	11	5^{b}
18	M	-1.6	95.1	Unknown	<50th	<50th	6^{b}	7^{b}

M: male, F: female, BMI: body mass index, SDS: standard deviation score, %: percentage, SBP: systolic blood pressure, DBP: diastolic blood pressure, BP: blood pressure, CAH: congenital adrenal hyperplasia.
a - BP >90th centile, b – Non-dipping BP

One child had evidence of raised systolic BP whilst awake (>90th centile but below 95th centile), and loss of systolic nocturnal dip. One child had evidence of raised diastolic BP (>90th centile but <95th centile), and loss of diastolic nocturnal dip. All ABPM measurements were in those with normal clinic BP. BP was higher in the younger participants who were not eligible for ABPM because of their age. One older child had hypertension on clinic BP measurement, but he did not tolerate ABPM.

4.3.3.4. Carotid intima media thickness (CIMT)

CIMT was performed on 21 participants (13 male) aged 12.0 (6.0 – 15.0) years. One child (aged 5 years) could not tolerate the procedure. One child could only tolerate one measurement.

Median CIMT of the average measurements was 0.41mm (0.4-0.44mm). Percentiles were generated for age and sex using reference data from a large cohort of healthy, normotensive children [162]. These are reported in Table 4.11.

Table 4.11: Distribution: Distribution of study participants by CIMT percentile

Percentile of CIMT measurement based on age and sex	**Number of participants with CIMT measurement within percentile category (n = 20)**
50th-75th	1 (5%)
75th-90th	13 (65%)
90th-95th	2* (10%)
>95th	4 (20%)

CIMT: carotid intima-media thickness, n: number.
* One of these participants had one measurement only

Further characteristics for those with CIMT measurements >95th centile for sex and age are shown in Table 4.12.

Table 4.12: Participants who had a CIMT >95th centile for age and sex

Participant	Age (years)	Sex (M/F)	Diagnosis	BMI SDS	Systolic BP percentile	Diastolic BP percentile	Fludrocortisone dose (mcg/m^2/day)	Hydrocortisone dose (mg/m^2/day)
005	11	M	Simple virilising CAH	1.6	89	93	-	15.7
011	11	M	Simple virilising CAH	3.3	94	95	-	5.6
014	6	M	Salt losing CAH	-0.4	90	83	230.3	11.1
016	12	M	Simple virilising CAH	3.7	91	28	-	6.8

M: male, F: female, BMI: body mass index, SDS: standard deviation score, BP: blood pressure, mcg: micrograms, mg: milligrams, CAH: congenital adrenal hyperplasia.

4.3.3.5. Flow mediated dilatation (FMD)

FMD was measured in 21 participants. Data were analysable in 18. Movement artefact in three young participants aged 5-10 years made analysis difficult and these data were excluded from the analysis. Results of FMD assessment can be seen in Table 4.13.

Table 4.13: Table showing FMD measured parameters in GRACE 1 participants

Measurement on ultrasound	Median (IQR)
FMD (%)	9.0 (4.4-12.3)
Baseline diameter (mm)	3.7 (3.2 - 4.4)
Maximum diameter (mm)	4.0 (3.5 – 4.9)
Time to maximum diameter (sec)	431 (388 – 459)

IQR: interquartile range, FMD: flow mediated dilatation, mm: millimetres, sec: seconds.

6/18 (33.3%) had an FMD of less than 7%. Two (11.1%) had FMD greater than 15% (18.2% and 25%). The remaining ten participants had FMD measurements between 7 and 15%, within the normal range. Those participants with FMD less than 7% are shown in Table 4.14.

Table 4.14: Table showing those participants with FMD measurements <7%

Participant	Age (years)	Sex (M/F)	Diagnosis	BMI SDS	Systolic BP percentile	Diastolic BP percentile	Fludrocortisone dose (mcg/m^2/day)	Hydrocortisone dose (mg/m^2/day)	CIMT centile
006	5	F	Salt losing CAH	-0.1	95	87	269.2	10.8	75^{th}-90^{th}
014	6	M	Salt losing CAH	-0.4	90	83	230.3	11.1	$>95^{th}$
015	7	F	3B-HSD deficiency & Barrter's)	-0.7	70	74	34.9	10.2	95^{th}
017	12	M	Simple virilising CAH	2.0	34	8	-	10.3	75^{th}
019	16	M	Addison's disease	1.0	46	4	102.6	10.3	75^{th}
024	16	M	Salt losing CAH (also has FH)	3.2	98	96	34.1	9.1	75^{th}-90^{th}

M: male, F: female, BMI: body mass index, SDS: standard deviation score, BP: blood pressure, mcg: micrograms, mg: milligrams, CIMT: carotid intima-media thickness, CAH: congenital adrenal hyperplasia, FH: familial hypercholesterolaemia.

4.3.4. Metabolic health

4.3.4.1. HOMA-IR

Average HOMA-IR was 1.94 ± 2.23. HOMA-IR values converted into centiles for age and sex and are detailed in Table 4.15[152].

Table 4.15: Distribution of study participants according to HOMA-IR percentile

Percentile of HOMA-IR measurement based on age and sex [152]	Number of participants with HOMA-IR measurement within percentile category (n = 26)
<3rd	6 (23.1%)
3-10th	3 (11.5%)
10-25th	2 (7.7%)
25th-50th	2 (7.7%)
50th-75th	2 (7.7%)
75th-90th	3 (11.5%)
90-97th	2 (7.6%)
>97th	6 (23.1%)

HOMA-IR: homeostatic model assessment for insulin resistance, n: number.

Studies used to formulate reference data have corrected for BMI. Eleven participants would be classed as over-weight/obese (≥85th percentile). Of those 11, none had a HOMA-IR that was raised for their increased BMI (Table 4.16).

Table 4.16: Table showing HOMA-IR percentiles for those participants who are overweight or obese (BMI>85th centile) compared to normative references ranges taking age, sex and BMI into account

Percentile of HOMA-IR measurement based on age and sex	Number of participants with HOMA-IR measurement within percentile category (n = 11)
<3rd	2 (18.2%)
3-10th	2 (18.2%)
10-25th	1 (9.1%)
25th-50th	2 (18.2%)
50th-75th	3 (27.3%)
75th-90th	1(9.1%)
>90th	0 (0%)

HOMA-IR: homeostatic model assessment for insulin resistance, n: number.

This may highlight that overweight/obese participants' BMI may explain their increase in HOMA-IR. Two participants had a normal weight (BMI<85th centile, actual BMI 17 and 19kg/m^2) and HOMA-IR >97th centile.

Table 4.17: Characteristics of study participants with HOMA-IR >97th centile according to age and sex

Participant	Age (years)	Sex (M/F)	Diagnosis	BMI SDS	Systolic BP percentile	Diastolic BP percentile	Fludrocortisone dose (mcg/m^2/day)	Hydrocortisone dose (mg/m^2/day)	CIMT centile	FMD measurement (%)
001	15	F	Addison's disease	1.89	13	20	100	22.5	75th	13.5
005	11	M	Simple virilising CAH	1.6	89	93	-	15.7	>95th	-
011	11	M	Simple virilising CAH	3.3	94	95	-	5.6	>95th	13.3
015	7	F	3B-HSD deficiency & Barrter's)	-0.7	70	74	34.9	10.2	95th	2.42
016	12	M	Simple virilising CAH	3.7	91	28	-	6.8	>95th	11.8
018	12	M	Addison's disease	1.4	68	65	107.1	14.3	75th	9.57
021	6	M	Salt losing CAH	0.7	99	92	174.4	8.7	75th	7.57
023	17	M	Salt losing CAH	2.3	76	55	35.7	9.5	75th-90th	14.19
025	13	M	Addison's disease	2.5	82	33	90.9	25.0	75th	8.63

M: male, F: female, BMI: body mass index, SDS: standard deviation score, BP: blood pressure, mcg: micrograms, mg: milligrams, CIMT: carotid intima-media thickness, FMD: flow mediated dilatation, %: percentage, CAH: congenital adrenal hyperplasia.

4.3.4.2. Plasma metanephrines

Plasma metanephrines and plasma normetanephrines were measured in participants. In 11 participants, plasma metanephrines were below the lower limit of detection for the assay (<(80pmol/L) and therefore it was not possible to determine whether the results lay within the reference range which extended below 80pmol/L [163]. All other metanephrine results were normal.

Two participants were found to have high metanephrines for their age (normotensive four- and five-year-old females with salt losing CAH, treated with fludrocortisone), whilst one four-year-old hypertensive (SBP >95th centile) male with salt-losing CAH, on fludrocortisone, had a low normetanephrine result. One participant's result was unable to be analysed. The rest were within normal limits. ABPM was not available for those participants with abnormal results. Plasma metanephrine concentrations did not correlate with BP in those treated with fludrocortisone.

4.3.4.3. Plasma leptin

Eight participants were aged six years and below, therefore their leptin concentrations were extrapolated for those aged six years based on reference data [158]. This affected 8 participants, which did not include anyone who had a leptin concentration >97.5th centile. Results can be seen in Table 4.18.

Table 4.18: Distribution of study participants according leptin concentration percentiles based on aged and gender. For those aged <6 years: 6-year-old equivalent reference ranges were used.

Percentile of Leptin measurement based on age and sex, using [158]	**Number of participants with plasma leptin measurement within percentile category (n = 26)**
<2.5th	1 (3.8)
2.5th-5th	1 (3.8)
5th-25th	7 (26.9)
25th-50th	5 (19.2)
50th-75th	0 (0)
75-95th	6 (23.1)
95th-97.5th	0 (0)
>97.5th	6 (23.1)

n: number.

All participants with a raised leptin had a BMI >90th centile, with 4/6 (66.6%) > 99th centile.

Table 4.19: Table showing participants with Leptin >97th centile for age and sex (shows participants with co-existing raised HOMA-IR)*

Participant	Age (years)	Sex (M/F)	Diagnosis	BMI SDS	Systolic BP percentile	Diastolic BP percentile	Fludrocortisone dose (mcg/m^2/day)	Hydrocortisone dose (mg/m^2/day)	CIMT centile	FMD measurement (%)
011	11	M	Simple virilising CAH	3.3	94	95	-	5.6	>95th	13.3
016	12	M	Simple virilising CAH	3.7	91	28	-	6.8	>95th	11.8
018	12	M	Addison's disease	1.4	68	65	107.1	14.3	75th	9.66
023	17	M	Salt losing CAH	2.3	76	55	35.7	9.5	75th-90th	14.22
024	16	M	Salt losing CAH (also has FH)	3.2	98	96	34.1	9.1	75th-90th	11.0
025	13	M	Addison's disease	2.5	82	33	90.9	25.0	75th	8.6

M: male, F: female, BMI: body mass index, SDS: standard deviation score, BP: blood pressure, mcg: micrograms, mg: milligrams, CIMT: carotid intima-media thickness, FMD: flow mediated dilatation, %: percentage, CAH: congenital adrenal hyperplasia, FH: familial hypercholesterolaemia

4.3.4.4. Comparison between leptin, HOMA-IR and obesity

Those participants with a raised HOMA-IR often had a rise in leptin. Both were associated with a rise in BMI. Correlations between leptin and HOMA-IR (r^2 0.83, $p<0.001$) and correlations between leptin and BMI (r^2 0.82, $p<0.001$) can be seen in Figure 4-6. Both insulin resistance and increased leptin concentrations are likely to be associated with a rise in BMI rather than a causal relationship. Neither HOMA-IR nor leptin concentrations correlated with BP.

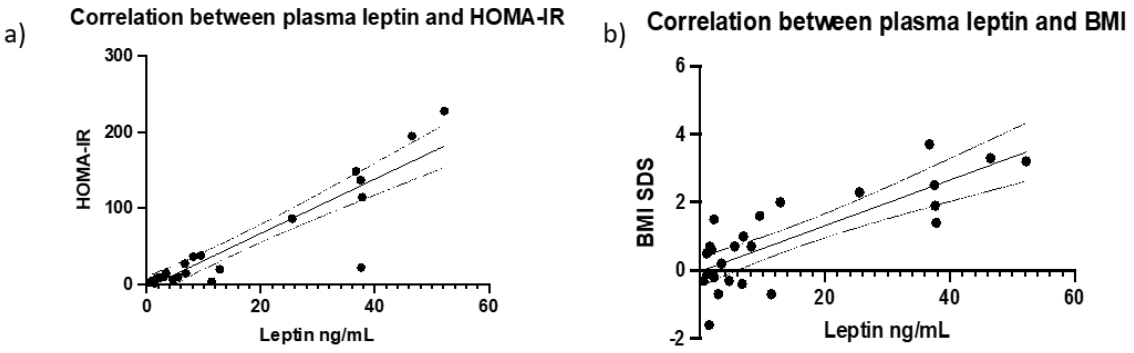


Figure 4-6: Graph showing a) correlation between plasma leptin concentrations and HOMA-IR and b) correlation between plasma leptin and BMI

4.3.4.5. Von Willebrand factor

Details relating to the relationship of VWF to cardiovascular disease is highlighted in section 1.5.1. The reference range used at Alder Hey for children aged two to 18 years is 55 – 145% for both VWF antigen and VWF activity. Median VWF activity was 91.4% (IQR 75.2 – 110%). Median VWF antigen was 96.7% (IQR 85.3 -115.4%). Three participants (11.5%) had evidence of low VWF activity, all with a normal VWF antigen level. After discussion with a consultant haematologist, as these participants have no history of bleeding, no clinical intervention is required. Three (11.5%) had evidence of raised VWF activity, two (66%) of whom also had a raised VWF antigen level. Participants details of those with raised VWF antigen and activity can be found in Table 4.20.

Table 4.20: Table showing participants with evidence of raised von Willebrand antigen and activity

Participant	Age (years)	Sex (M/F)	Diagnosis	BMI SDS	Systolic BP percentile	Diastolic BP percentile	Fludrocortisone dose (mcg/m^2/day)	Hydrocortisone dose (mg/m^2/day)	CIMT centile	FMD measurement (%)
015	7	F	3B-HSD deficiency & Bartter's)	-0.7	70	74	34.9	10.2	95th	2.42
024	16	M	Salt losing CAH (also has FH)	3.2	98	96	34.1	9.1	75th-90th	0.97
025	13	M	Addison's disease	2.5	82	33	90.9	25.0	75th	8.63

M: male, F: female, BMI: body mass index, SDS: standard deviation score, BP: blood pressure, mcg: micrograms, mg: milligrams, CIMT: carotid intima-media thickness, FMD: flow mediated dilatation, %: percentage, CAH: congenital adrenal hyperplasia, FH: familial hypercholesterolaemia.

4.3.5. Salivary cortisol, cortisone and adrenal biomarkers

Salivary cortisol, cortisone, 17OHP, testosterone, A4, 11KT and 11OHA4 were measured at the study visit and two-hourly thereafter. An early morning sample was not initially performed. On realisation of this, a substantial ethical amendment was submitted to request an early morning (prior to hydrocortisone dose) sample and a sample two hours afterwards. The rest of the samples were not timed with hydrocortisone dosing to give an overall trend throughout the day with an aim to understand overall cortisol and cortisone exposure compared to that of healthy children.

Salivary sampling was available for all four participants with Addison's disease. All were pubertal, three were male. Fifteen patients with CAH provided salivary samples (two males <10 years, six males ≥ 10 years, five females <9 years and two females ≥ 9 years). The one participant with primary AI of unknown origin is described separately. For salivary cortisol and cortisone, the whole population was compared to all children from the normative data. For other adrenal biomarkers (testosterone, A4, 11KT and 11OHA4) study participants were compared against randomly matched controls for age and sex from the cohort of healthy children and young people described in chapter 3. One participant did not collect salivary samples (male aged two years). Five participants had insufficient samples. Graphs are presented as median and 95% CI. All statistical tests compare samples at two to twelve hours in both the control group and the group with primary AI to allow for lack of early morning sampling in the cohort with AI.

4.3.5.1. Salivary cortisol

Salivary cortisol measurements were available in 25 participants. Possible contamination of salivary samples occurred. Values >230nmol/L were removed. These samples ranged from 438.6nmol/L to 5193.0nmol/L. Median salivary cortisol in the control group was 2.2nmol/L and 17.1nmol/L in the group with primary AI. There was a significant difference of p=0.002 (see Figure 4-7).

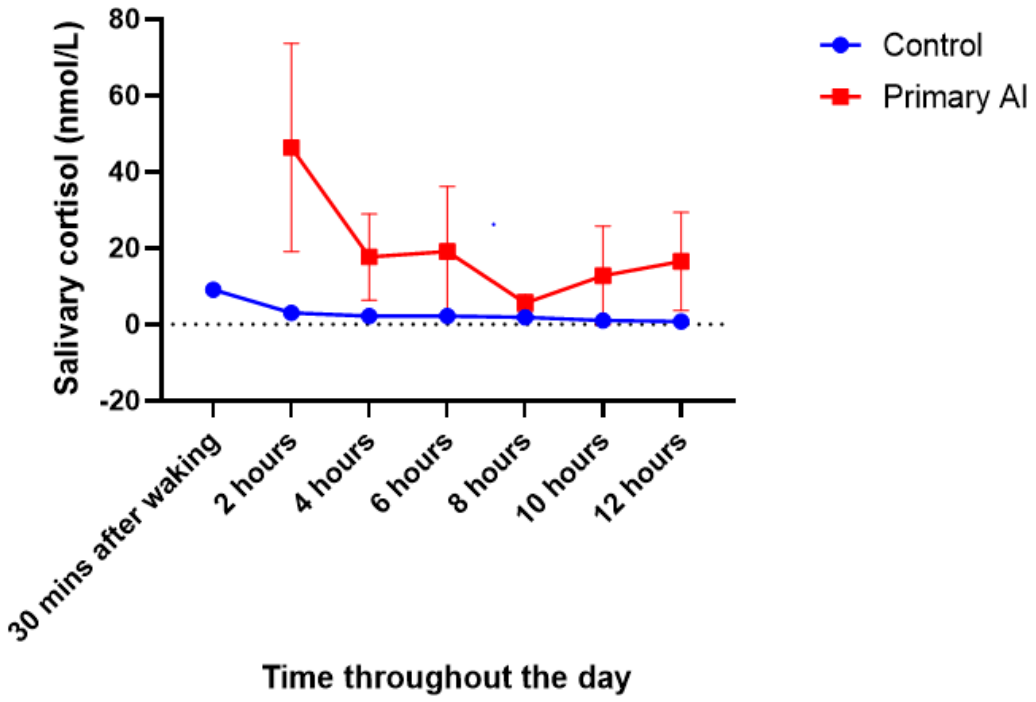


Figure 4-7: Graph showing salivary cortisol (nmol/L) in participants with primary AI, treated with hydrocortisone compared to a cohort of healthy children

There was concerns that some salivary samples remained contaminated with hydrocortisone therefore, using the normative data any value that was twice the upper limit of normal was removed (any value >78nmol/L). The comparison can be seen below (Figure 4-8). The difference remains significant with mean of 14.6nmol/L in primary AI compared to 2.2nmol/L in the healthy cohort, p=0.004. AUC of salivary cortisol did not correlate with hydrocortisone dose. Individual salivary cortisol profiles can be seen in appendix 9.6.

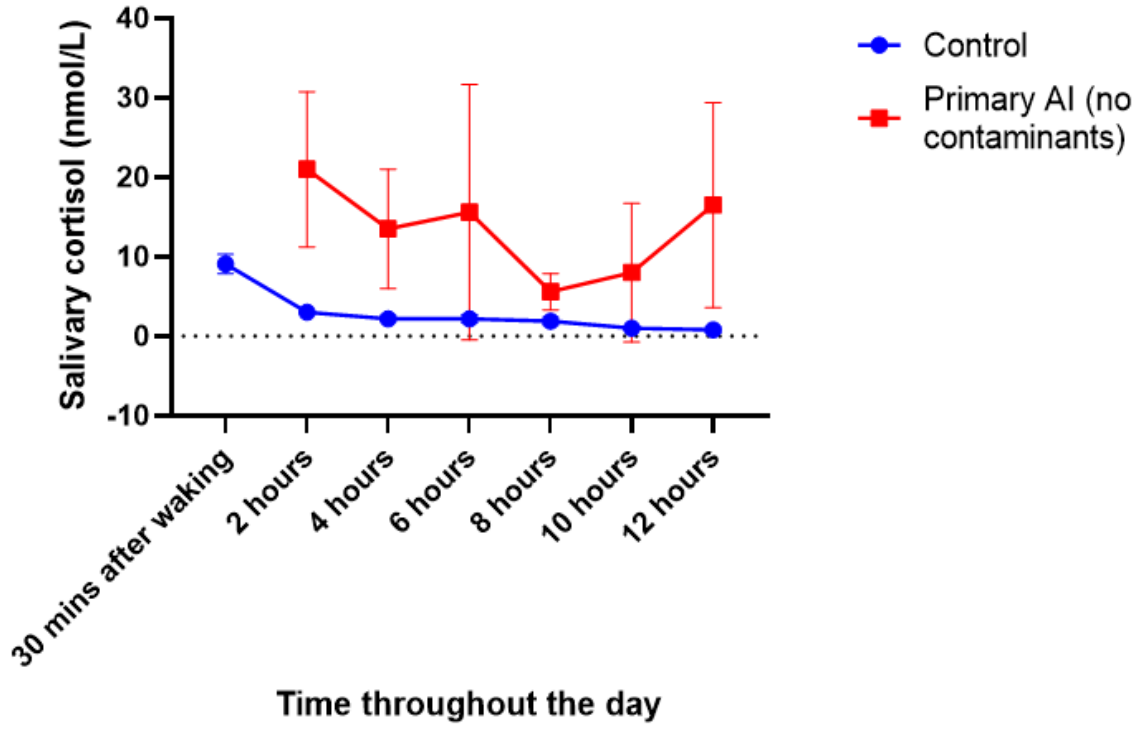


Figure 4-8: Graph showing salivary cortisol with all likely contaminants removed in participants with primary AI, treated with hydrocortisone compared to a cohort of healthy children

4.3.5.2. Salivary cortisone

Salivary cortisone concentrations can be seen in Figure 4-9. The median salivary cortisone concentration was 14.1nmol/L in the healthy cohort with a median salivary cortisone concentration of 11.4nmol/L in the participants with primary AI, p =0.63.

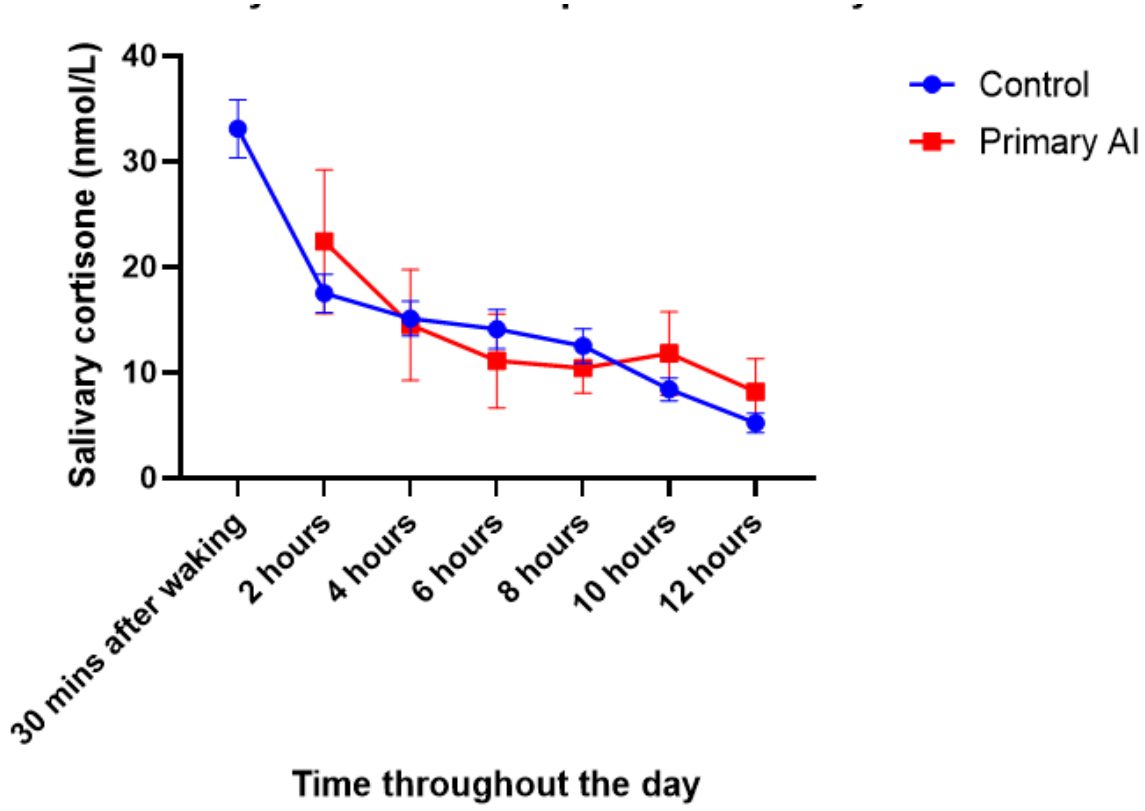


Figure 4-9: Graph showing salivary cortisone concentrations (nmol/L) in participants with primary AI, treated with hydrocortisone compared to a cohort of healthy children

The differences between salivary cortisone in those with CAH and Addison's disease compared to their matched controls can be seen in Figure 4-10 and Figure 4-11. Neither showed a statistically significant difference in cortisone profiles. AUC of salivary cortisone and the study visit sample at 9am did not correlate with hydrocortisone dose. Individual salivary cortisone profiles can be seen in appendix 9.6.

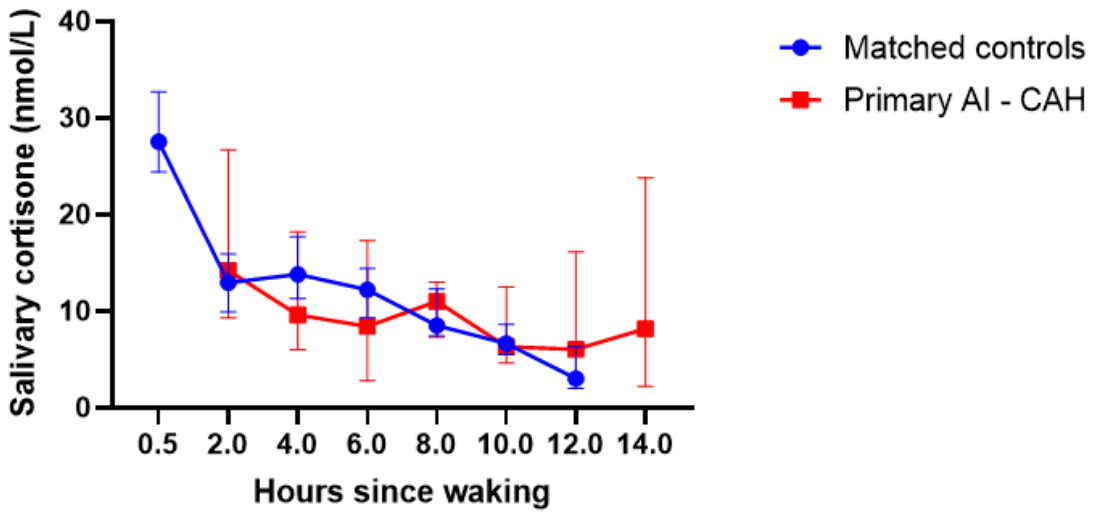


Figure 4-10: Salivary cortisone (nmol/L) in participants with CAH (n=21) compared to matched healthy controls

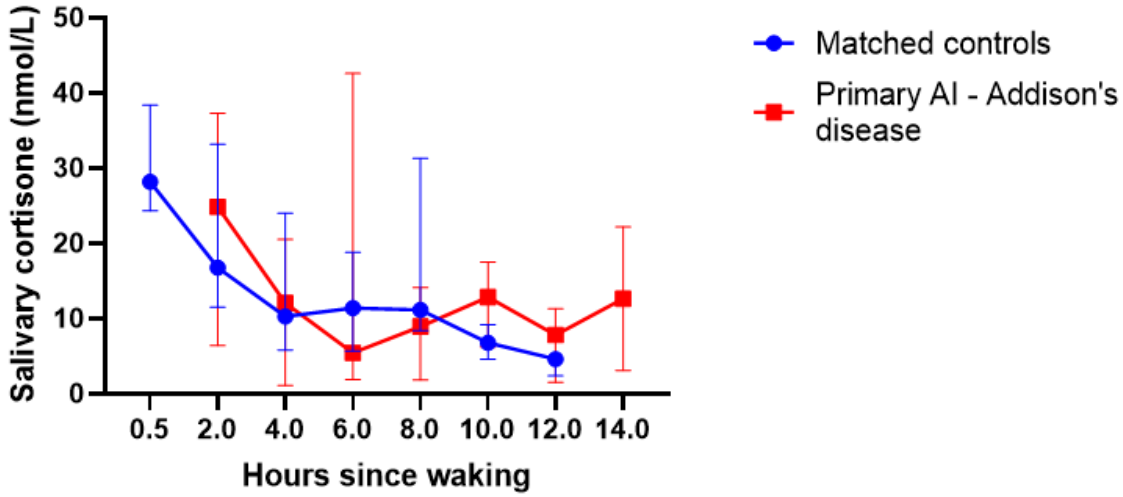


Figure 4-11: Salivary cortisone (nmol/L) in participants with Addison's disease (n=4) compared to matched healthy controls

4.3.5.3. Salivary cortisone: cortisol ratio

The salivary cortisone: cortisol ratio also showed a significant difference with a median value of 8.4 in the healthy cohort and 2.95 in participants with primary AI, p=0.002 (Figure 4-12).

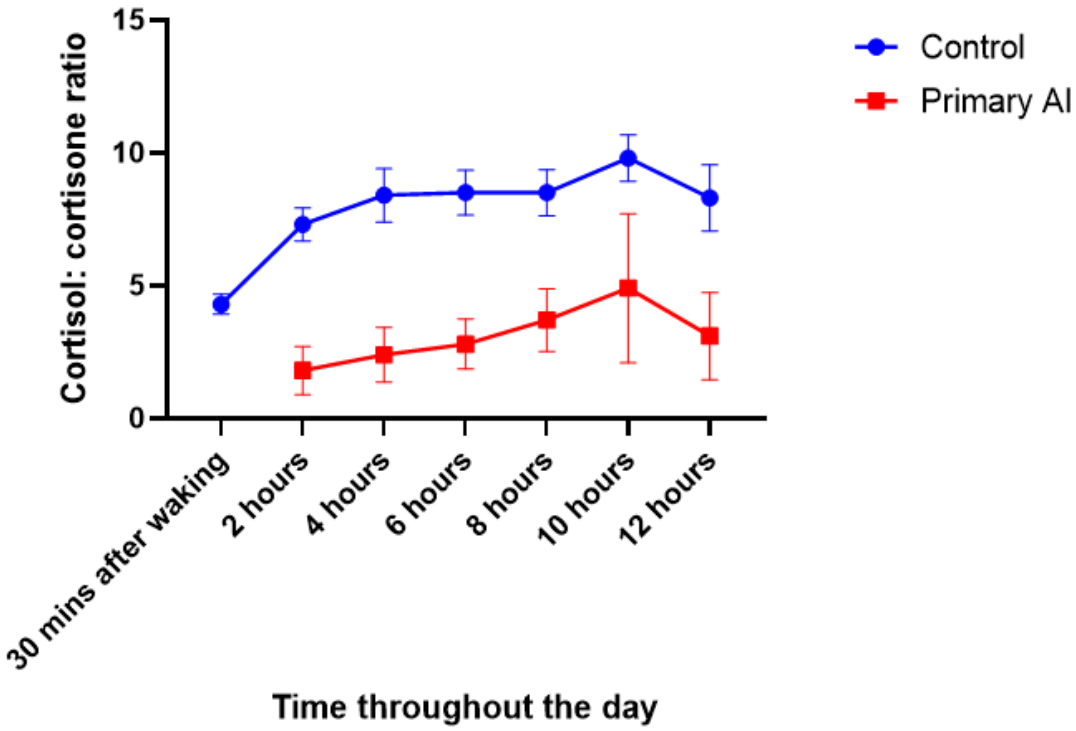


Figure 4-12: Graph showing salivary cortisone: cortisol ratio in participants with primary AI treated with hydrocortisone compared to a cohort of healthy children

AUC for salivary cortisol and salivary cortisone did not correlate with blood pressure, mean glucose on CGMS or HOMA-IR. Salivary cortisol and cortisone could not be analysed in periods of hypoglycaemia as salivary sampling was taken in the first 24 hours. CGMS data from the first 24 hours were excluded. ABPM data showed an average over the 24 hours therefore period of hypotension or hypertension could not be depicted at sampling times.

4.3.5.4. Salivary 17OHP

Salivary 17OHP concentrations were analysed in the participants with primary AI. Salivary 17OHP concentrations were not available in the cohort of healthy children. 17OHP concentrations by diagnoses of Addison's disease, primary AI of unknown aetiology and CAH

can be seen in Figure 4-13. Concentrations throughout the day can be seen in Table 4.21.

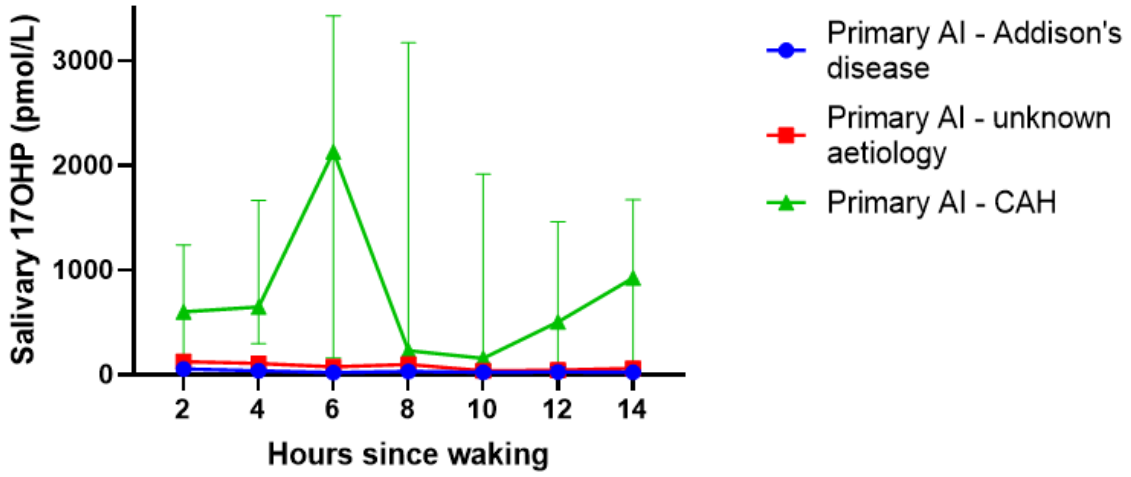


Figure 4-13: Graph showing salivary 17OHP concentrations (pmol/L) in participants with Addison's disease, primary AI of unknown aetiology and CAH

Table 4.21: Table showing median, 5th and 95th centiles for salivary 17OHP concentrations in participants with Addison's disease, primary AI of unknown aetiology and CAH

Time since waking (hours)	Salivary 17OHP (pmol/L) in Addison's disease*, median (5th and 95th centile)	Salivary 17OHP (pmol/L) in primary AI – unknown aetiology	Salivary 17OHP (pmol/L) in CAH**, median (5th and 95th centile)
2	54 (51.7 – 26.0)	123	780.7 (69.1 – 4707.2)
4	35 (18.4 – 56.1)	106	588.4 (79.7 – 6838.7)
6	33 (20.3 – 45.6)	73	2164.7 (74.5 – 9116.7)
8	36 (28.4 – 44.2)	99	212.8 (72.2 – 5821.9)
10	40 (33.1 – 46.6)	36	162.1 (48.2 – 8238.3)
12	24 (15.2 – 63.4)	43	50.3.6 (51.3 – 9348.1)

17OHP: 17-hydroxprogesterone, pmol/L: picomoles per litre, AI: adrenal insufficiency, CAH: congenital adrenal hyperplasia.
*5/26 samples undetectable, 2 samples insufficient in Addison's disease group
**43/118 samples unable to be analysed as insufficient or evidence of interference

4.3.5.5. Salivary testosterone

Salivary testosterone (pmol/L) in participants with Addison's disease tended to be higher but not significantly so, using Wilcoxon matched-pairs signed rank test. This is likely explained by the small numbers and the large 95% confidence intervals in the two groups (Figure 4-14).

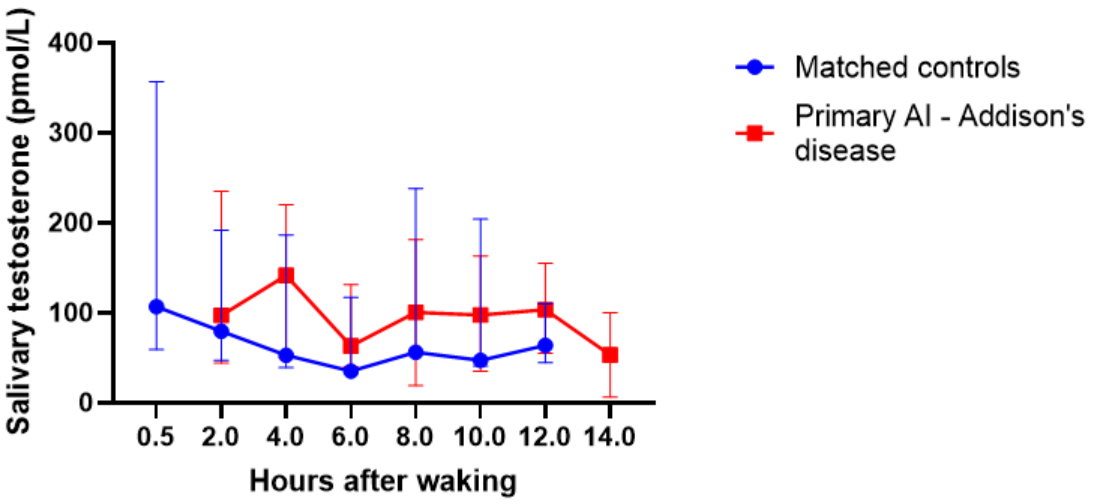
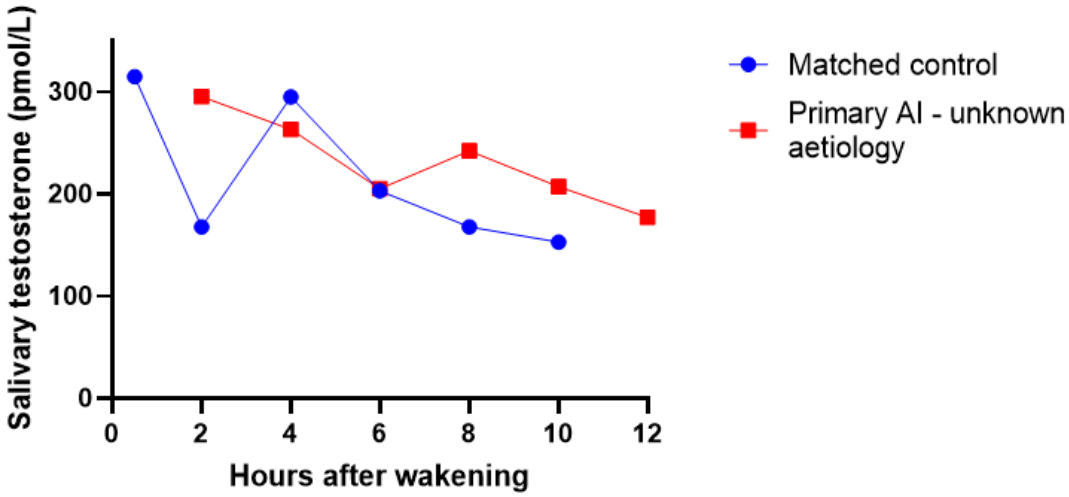


Figure 4-14: Salivary testosterone (pmol/L) in participants with Addison's disease compared to matched healthy controls.

Salivary testosterone (pmol/L) in the child with primary AI of unknown aetiology can be seen compared to a matched control. No statistical test was performed on these samples as there was only one participant.



A statistically higher salivary testosterone (pmol/L) can be seen between participants with CAH compared to matched healthy controls, p=0.03 using the Wilcoxon matched-pairs signed rank test.

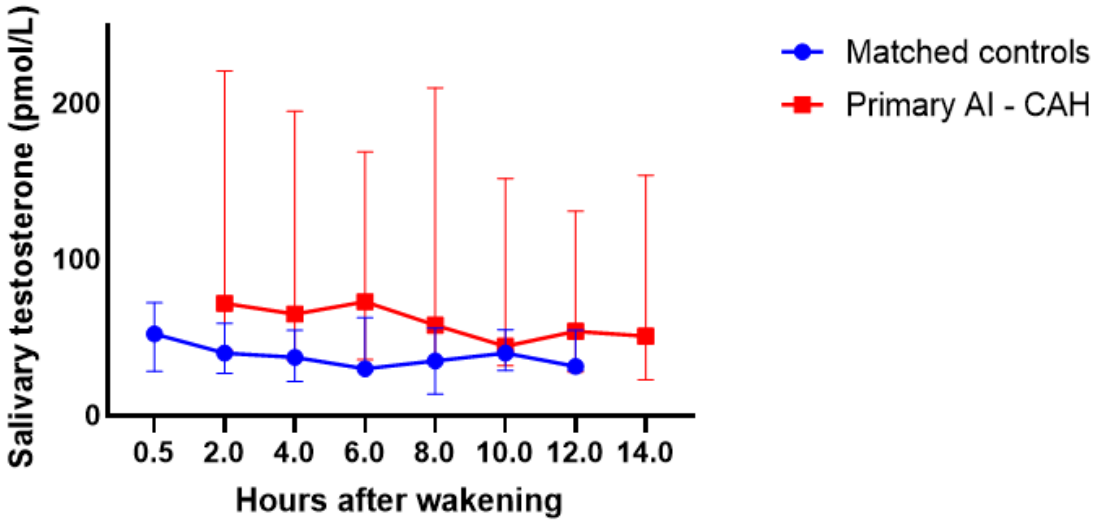


Figure 4-15: Salivary testosterone (pmol/L) in participants with CAH compared to matched healthy controls.

4.3.5.6. Salivary androstenedione

Salivary A4 (pmol/L) was increased in participants with Addison's disease (p=0.03) compared to matched healthy controls (Figure 4-16). Salivary A4 was also higher in the participant with AI of unknown aetiology compared to a matched healthy control. Again, no statistical test was performed for this one participant (Figure 4-17). Salivary A4 was also increased in those with CAH, p=0.03 (Figure 4-18).

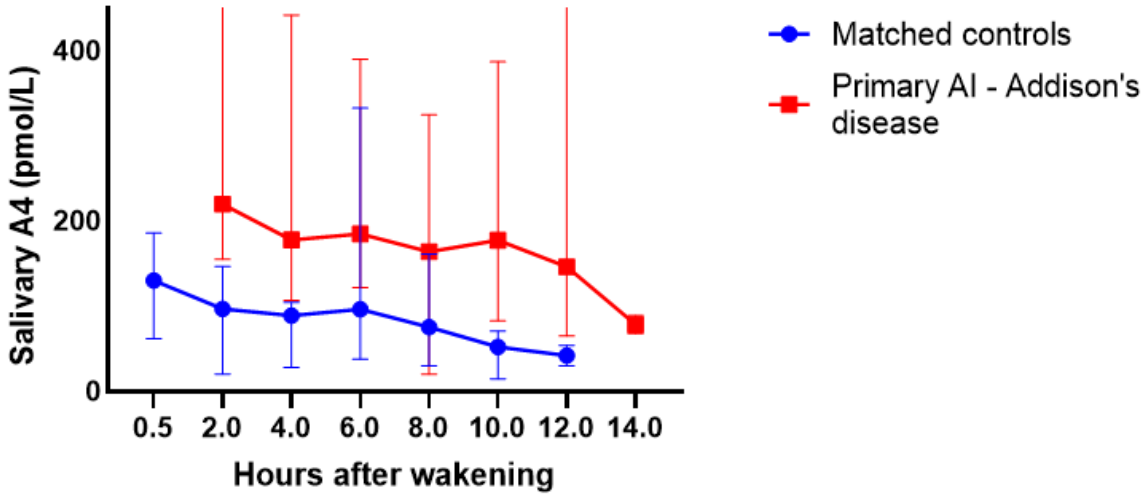


Figure 4-16: Salivary A4 (pmol/L) in participants with Addison's disease compared to matched healthy controls

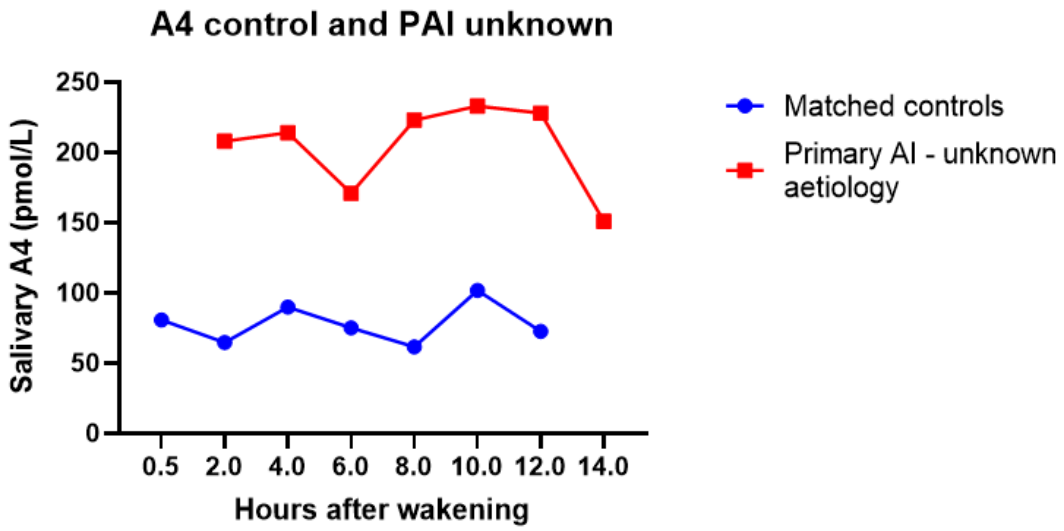


Figure 4-17: Salivary A4 (pmol/L) in participant with primary AI of unknown aetiology compared to a matched control.

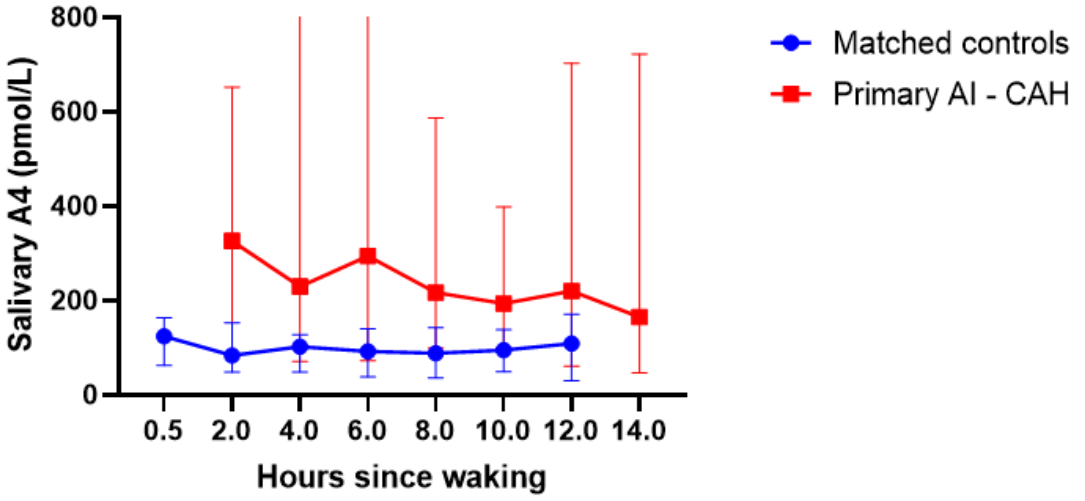


Figure 4-18: Salivary A4 (pmol/L) in participants with CAH compared to matched healthy controls

4.3.5.7. Salivary 11KT

Salivary 11KT was low in participants with Addison's disease compared to matched healthy controls (p=0.03). Figure 4-19 shows participants with Addison's disease had very low (mostly undetectable) concentrations of salivary 11KT. Of the 24 samples salivary 11KT was undetectable in 22. The further two samples had concentrations of 6.1 and 7 pmol/L. Twenty-five samples were available for matched controls ranging from 47.7 – 272.6pmol/L. No healthy children had salivary samples that showed undetectable concentrations of 11KT. The

participant with primary AI of unknown aetiology showed similar low concentrations of 11KT (Figure 4-19). Salivary 11KT was also higher in participants with CAH compared to healthy controls (p=0.03) (Figure 4-21).

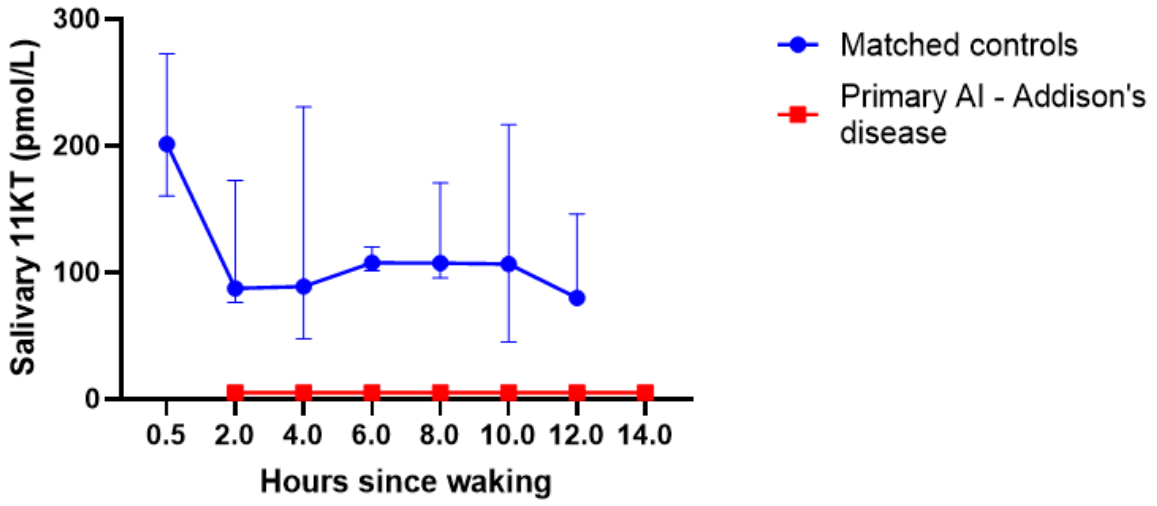


Figure 4-19: Salivary 11KT (pmol/L) in participants with Addison's disease compared to matched healthy controls

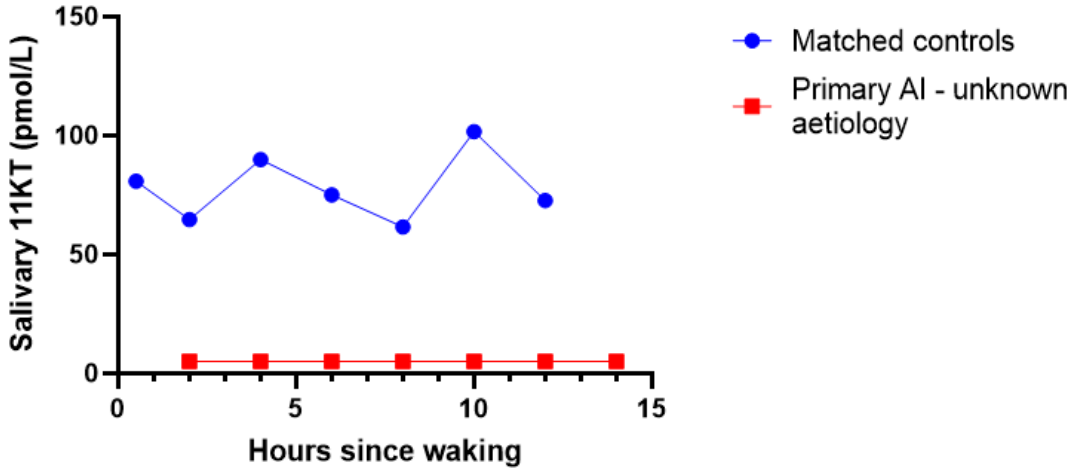


Figure 4-20: Salivary 11KT (pmol/L) in participant with primary AI of unknown aetiology compared to a matched healthy control

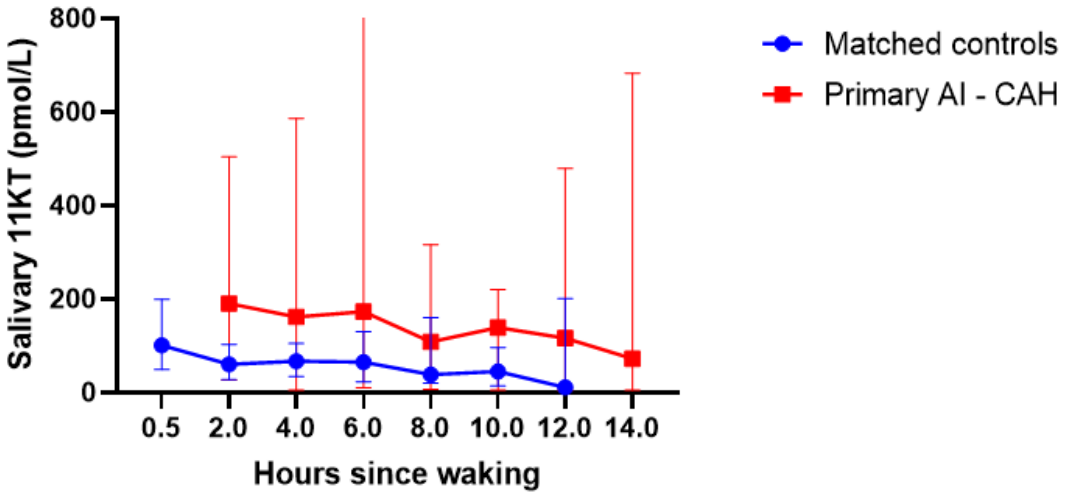


Figure 4-21: Salivary 11KT (pmol/L) in participants with CAH compared to matched healthy controls

4.3.5.8. Salivary 11OHA4

Salivary 11OHA4 concentrations were similar between participants with Addison's disease and matched controls. In the participants with Addison's disease 11OHA4 was undetectable in 20 out of 24 samples. 11OHA4 was undetectable in 16 out of 25 samples in the matched controls (Figure 4-22). Three of the four participants with Addison's disease are post pubertal males. Table 3.7 shows that undetectable concentrations of 11OHA4 was not unusual in the healthy population. The lower level of detection is 45pmol/L. Figure 4-23 shows the participant with primary AI of unknown aetiology compared to a matched, healthy control. Participants with CAH had significantly higher salivary 11OHA4 concentrations compared to healthy controls matched for age and sex (Figure 4-24).

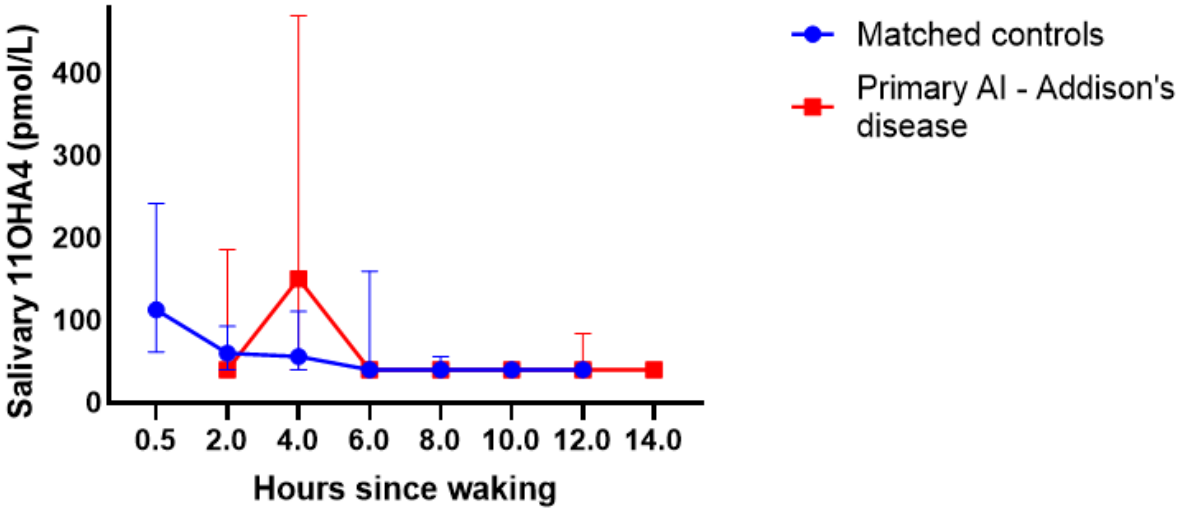


Figure 4-22: Salivary 11OHA4 (pmol/L) concentrations in participants with Addison's disease compared to healthy controls

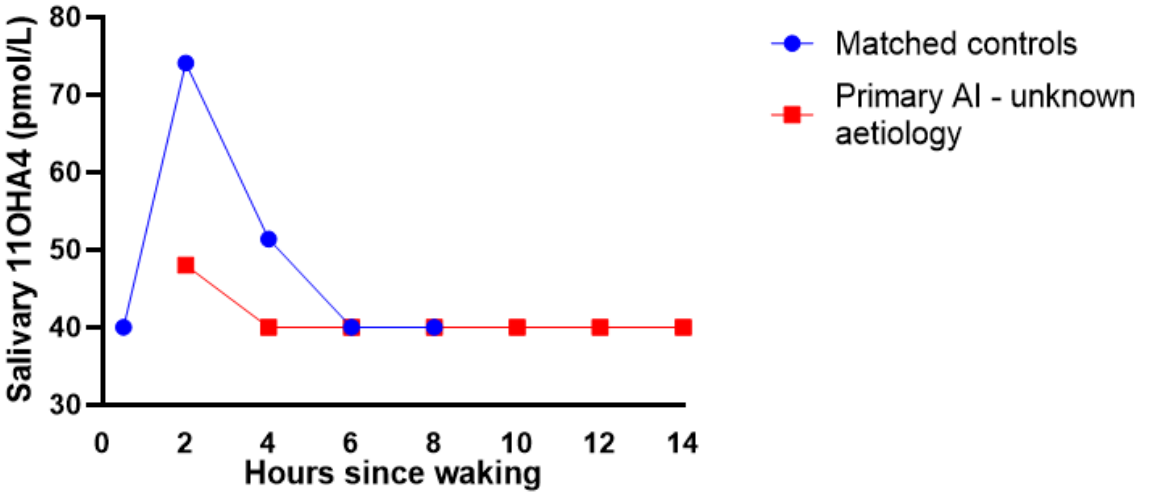


Figure 4-23: Graph showing salivary 11OHA4 (pmol/L) concentrations in the participant with primary AI of unknown aetiology compared to a matched healthy control

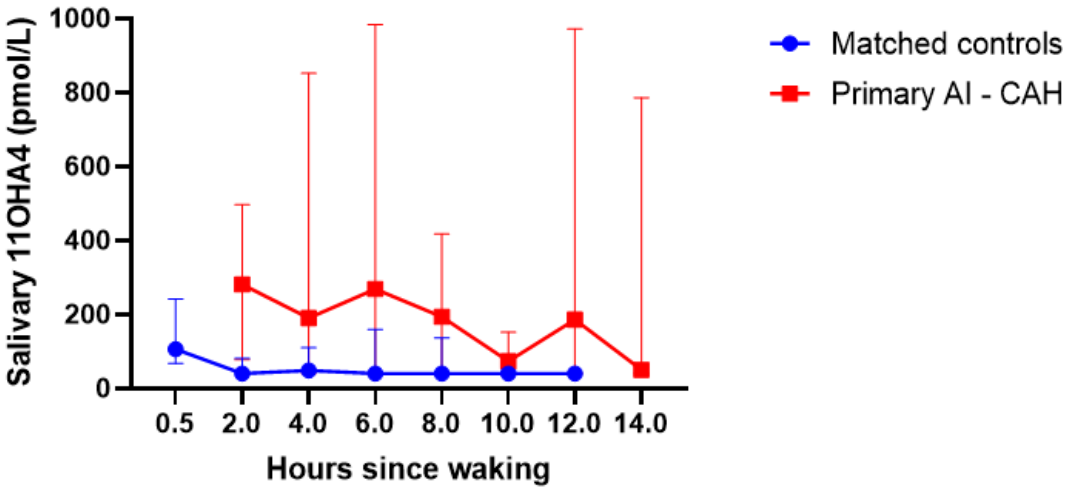


Figure 4-24: Graph showing salivary 11OHA4 (pmol/L) concentrations in participants with CAH compared to matched healthy controls.

4.4. Discussion

This study has contributed the first large dataset assessing glucose concentration via CGMS over a one-week period in children and young people with primary AI, showing that higher mean glucose concentrations are more common than prolonged periods of hypoglycaemia. Secondary outcomes show that children and young people have evidence of cardiovascular risk factors such as obesity, hypertension, increased CIMT, reduced FMD and raised biochemical markers of cardiovascular and metabolic risk. Salivary cortisol is not helpful for monitoring in children taking hydrocortisone treatment as contamination is likely. Salivary cortisone is likely to be a better marker of cortisol status. Salivary testosterone and androstenedione are higher in both Addison's disease and CAH, whilst 11KT and 11OHA4 are lower in Addison's disease but higher in children and young people with CAH.

4.4.1. Auxology

This study population has a normal distribution for height centred at approximately 0 SDS. This is reassuring showing that height does not seem to have been suppressed or exaggerated and remains within limits set in the general population. Reduced final adult height in patients with CAH has previously been described [164]. Approximately half of the cohort are pre-pubertal (estimated for age as no tanner staging took place). It is unknown as to whether control of androgens during puberty has the biggest impact on final adult height. BMI SDS seems to be higher than SDS scores would recommend. It is unknown as to whether BMI SDS scores found here reflect the increasing BMI that is being seen within the general population [165]. It is well reported that having CAH is associated with increased BMI [166]. GRACE 1 data support these findings.

4.4.2. Glucose regulation

The primary outcome for GRACE 1 assessed whether there was evidence of hypoglycaemia in children and young people with primary AI. Interestingly, glucose concentrations tended to be higher in this population, compared to data collected in healthy children [38]. One of the twenty-six participants with primary AI did show evidence of hypoglycaemia. On adjusting his hydrocortisone dosing (9.2 to 9.8mg/m^2/day) his interstitial fluid glucose levels improved (from spending 2.4% of time with glucose <3mmol/L to 1.1% of the time). Hypoglycaemia occurred over night and throughout the day. Prior to study involvement, this participant had suffered from an adrenal crisis, requiring glucagon in the early morning, with no known

precipitant. This would suggest that hypoglycaemia may be a possibility and clinical concerns should be addressed accordingly. Interestingly, no participants had a fasting glucose ≤ 3.2 mmol/L on the morning of the first study visit. Participants had fasted for at least 10 hours at the time of sampling, and many for longer than this. All had taken their dose of hydrocortisone. This is reflected in the work by Johnstone et al [66] who showed that no child with ACTH deficiency became hypoglycaemia if they had taken their hydrocortisone. It was only children who had missed their hydrocortisone dose that had become hypoglycaemic. As expected, hydrocortisone treatment seems protective against hypoglycaemia.

Interestingly both Cambiaso et al [65] and Watanabe et al [68] used CGMS monitoring for 24-48 hours after the sensor had been inserted. In our study cohort, there were periods of hypoglycaemia that were in fact artefact during this time. It is likely that the sensor sat within a small area of blood surrounding the sensor after it had been inserted that led to false readings of hypoglycaemia. It was these inaccuracies that meant that the first 24 hours of data were removed both from the GRACE 1 population data and the raw data from healthy children.

Children with primary AI tended towards a higher mean glucose, with evidence of glucose concentrations >7.8mmol/L for a higher percentage of time than healthy control subjects, although not statistically significant when a Bonferroni correction was used. The clinical significance of these higher glucose concentrations is unknown. However, it has been reported that even slightly higher (>7.8mmol/L) in otherwise healthy adults can lead to an increase in cardiovascular risk [167]. Even slightly higher glucose concentrations may lead to increased risk, particularly in children who have adrenal insufficiency and are treated with hydrocortisone throughout their lifetime. Interstitial fluid glucose concentrations >8.9mmol/L or 10mmol/L were not significantly different suggesting that glucose concentrations were only slighter higher, rather than concentrations that would near a diagnosis of diabetes.

Hyperglycaemia may occur when cortisol concentrations peak post dose. Standard deviation of glucose measurements did not differ between groups. Those participants with higher glucose concentrations tended towards higher glucose concentrations for an increased amount of time, whilst those with lower glucose

concentrations tended towards them for an increased amount of time. This would discount a hypothesis that glucose variability was increased in children with primary AI taking hydrocortisone. Glucose concentrations tended to be higher or lower than normative data rather than evidence of hyper and hypoglycaemia and increased variability within one participant.

Primary AI has been associated with an increased incidence of insulin resistance [4], which has been replicated in this study based on HOMA-IR values. It is currently unknown as to when glucose concentrations start to rise prior to the onset of diabetes. This may be seen earlier than initially thought. Long term, increased glucose concentrations may serve as an additional cardiovascular risk factor.

4.4.3. Cardiovascular outcomes

Fludrocortisone dosing seems to influence BP more than hydrocortisone dosing. Hydrocortisone doses, in this cohort, were not excessive compared to other studies describing hydrocortisone dosing in children and young people with CAH, which may be as high as 15 – 25 mg/m^2/day. Doses of fludrocortisone were higher in the younger population when calculated for body surface area. It is known that there may be an element of aldosterone resistance during infancy [168]. As infants grow to become toddlers, fludrocortisone doses should be reduced to reduce the risk of hypertension observed in the younger pre-school children. Locally, we routinely use annual renin measurements to guide fludrocortisone dosing. It is possible that focussing on clinic BP measurements may be more beneficial. An important aspect of this would be to ensure that BP measurements taken in clinic would be converted to percentiles according to age, sex and height.

ABPM was difficult to tolerate for this cohort of patients. Participants had a median age 10.0, ranging from 2.0 – 18.0 years, with 13 (50%) of participants aged less than 10 years. ABPM was not performed in children aged less than seven years, a clinical decision was made for those between 7-10 years, whilst those aged 10 years and over were given an ABPM monitor with varying degrees of success. Only 9/13, of those eligible, tolerated ABPM, with greater than 50% successful readings. European guidance suggests that children should have >80% of readings successful before interpreting 24-hour profiles [161]. For the purpose of this thesis, we reported those children with more than 50% successful readings. BP is measured every 30 minutes in the day and every hour at night. Some children and young people could tolerate it

in the day but removed it on going to bed. This is a recognised difficulty in the adult population [169]. It is for this reason that ABPM is not widely used in paediatrics, unless there is a significant clinical need.

Participants in this cohort also had evidence of other cardiovascular risk factors including increased CIMT and reduced FMD indicating early signs of endothelial dysfunction. CIMT measures the thickness of the carotid intima-media, which is a recognised risk factor for cardiovascular morbidity and mortality [170]. Normative data sets have been described in children [162, 171]. Data for local healthy controls would be an ideal comparison, however, in the absence of control subjects, normative paediatric data has been used to interpret results, taking into consideration the effect of age and gender. CIMT percentiles, rather than absolute measurements in millimetres were reported. Within this cohort, increased CIMT values can already be seen. This is in keeping with previous literature [97, 172], showing increased CIMT values in children and young people with CAH. This has not, previously, been described in children and young people with Addison's disease. No participant with Addison's disease had an increased CIMT measurement for age and sex.

FMD is a measurement of endothelial function, and a surrogate marker for cardiovascular disease risk [173, 174]. FMD was abnormal in 6/18 (33.3%) of participants, suggesting that endothelial dysfunction may be present from an early age. All children with FMD measurement of less than 7% had a CIMT measurement greater than the 75th percentile for age and sex. BP was also greater than the 90th centile in 50% of those with low FMD readings. These findings suggest clustering of cardiovascular risk factors from an early age in children and young people with primary AI. Children with CAH (n =5/21) and Addison's disease (n = 1/4) both showed signs of endothelial dysfunction using FMD as the marker of disease.

High VWF levels are reported to reflect endothelial dysfunction [83, 84]. VWF is involved in platelet aggregation. It is stored in endothelial cells and thought to be released when there is evidence of dysfunction. High antigen and activity can also occur in times of stress [175]. It is possible that VWF levels have increased in these participants secondary to stress of the study visit or of venepuncture. VWF antigen and activity were high in children with other cardiovascular risk factors. 2/3 (66.6%) had a raised BMI SDS (>2.5 SDS), 1/3 (33.3%) was hypertensive with a BP >95th centile, all had CIMT >75th centile and 2/3 (66.6%) had evidence

of reduced FMD. VWF may be an additional marker of cardiovascular health in children and young people with CAH, highlighted by its consistency with other additional risk factors.

4.4.4. Metabolic outcomes

Metabolic risk factors are also raised in this population. HOMA-IR reference ranges depend on age and sex to calculate them. However, all references consider obesity when calculating HOMA-IR. In this study population, it seems to be reasonable to compare HOMA-IR to the general population, rather than accounting for obesity. The reason being that both a raised HOMA-IR and obesity serve as two linked risk factors associated with morbidity. Both risk factors lead to increased risk of cardiovascular disease and therefore both weight and insulin resistance should be addressed to reduce disease burden, although improvements in weight should also reduce insulin resistance [176]. Healthy diet and exercise can be used to manage both metabolic concerns. Leptin levels were high in all patients with a raised HOMA-IR which may also reflect BMI.

4.4.5. Salivary cortisol and cortisone

Salivary cortisol concentrations are higher in children with primary AI, treated with hydrocortisone compared to the cohort of healthy children. Whilst undetectable salivary cortisol concentrations can be found in the normative population, contaminants can be found in those on hydrocortisone treatment. To mitigate this risk, each value that was double the greatest mean (78nmol/L) were excluded from analysis. The difference between cohorts remained statistically significant. This may not have excluded every contaminant and this needs to be considered when interpreting the results. Time points mimicked those taken in the healthy cohort, sampling two-hourly throughout the day. Waking samples are awaited as part of the amendment to the GRACE 1 study. Thus far, 11 of the 26 participants have returned their samples. One child now takes Efmody®, a MR-HC. A further child is now treated with prednisolone and therefore was excluded from further analysis. Because of the lack of early morning cortisol in the primary AI population comparisons were made from two- to twelve-hour sampling within the healthy cohort. Graphs show the early morning sample for completeness (Figure 4-7). Error bars cannot be seen in the healthy cohort whilst error bars are much larger in the GRACE 1 population. Doses were taken at the times that had been clinically prescribed. Some samples will be trough and some samples will be peak concentrations, which will explain the high variability. All participants were asked not to

perform their saliva sampling within one hour of a dose to reduce contamination as much as possible.

There was no statistical difference in salivary cortisone between the two groups. This may reflect normative cortisone concentrations in participants on maintenance or replacement doses of hydrocortisone. A normal physiological secretion of approximately 8mg/m^2/day has been demonstrated [177]. This cohort of participants with primary AI received 9.8 (IQR 9.0 – 10.8) mg/m^2/day of hydrocortisone. Similar salivary cortisone concentrations between this group suggest that treatment is at physiological replacement doses. Alternatively, 11β-HSD activity may have reached its capacity and therefore free cortisone cannot pass into the saliva at concentrations higher than this threshold. This is unlikely as the saturation of CBG occurs before the saturation of 11βHSD enzymes and therefore salivary cortisone concentrations would be expected to reflect serum cortisol even more closely at the higher concentrations. Unsurprisingly, considering that salivary cortisol concentrations were much higher in the treated group, there was also a significant difference in the salivary cortisone: cortisol ratio.

Early morning salivary concentrations of both cortisol and cortisone in the children with primary AI would be interesting as there should be limited contamination at this time. These samples are unlikely to affect clinical care as salivary concentrations would be expected to be low at this time, to reflect the lack of endogenous secretion of cortisol in this condition but would be useful for further research in the future.

4.4.6. Salivary androgens

All four salivary androgens mimic the diurnal rhythm of cortisol and cortisone. These samples were collected from approximately 9am at the study visit then two-hourly throughout the day. Further samples have been requested for before the first dose of hydrocortisone in the morning and two hours afterwards. These results are not currently available.

4.4.6.1. Salivary androgens in Addison's disease

Salivary testosterone concentrations tend to be higher in participants with Addison's disease compared to healthy children, although this is not statistically significant. Only four participants have Addison's disease who are all post pubertal, three are male, one female. Each participant provided five to seven samples. Twenty-five samples were analysable. Matched controls for age and sex were used to compare. This increased salivary testosterone concentration would be unexpected as the hypothalamic-pituitary-gonadal (HPG) axis is not

thought to be affected in Addison's disease. However, this may be secondary to loss of negative feedback of 11KT. 11KT is a potent androgen, thought to be as potent as testosterone and dihydrotestosterone regarding its effects on the androgen receptor [178]. 11KT is undetectable on all samples except two in participants with Addison's disease. The two samples were 11KT is detectable only reach 6.1 and 7.0pmol/L. 11KT is derived from the adrenal gland. These findings support this theory as Addison's disease leads to destruction of the adrenal gland from adrenal antibodies. It is unlikely that high concentrations are made within the gonad, otherwise 11KT would be thought to be higher is this disease. It may be that the loss of negative feedback from 11KT leads to increased salivary free testosterone in the HPG pathway. Alternatives theories could be that the rise in ACTH would lead to increased activation of the HPG axis. However, receptors are not similar therefore this may be more unlikely.

Further research is required to support these findings and determine the reason for this rise. Raised salivary A4 concentrations are also found in Addison's disease. The reason for this is unknown. Salivary 11OHA4 concentrations are very similar between those with Addison's disease and healthy controls. Healthy controls have low concentrations of 11OHA4, often undetectable and this is reflected in the participants with Addison's disease. 11OHA4 is also thought to be derived from the adrenal gland. Again, this is supported with a direct insult of the adrenal gland seen in Addison's disease. There was only one participant with primary AI of unknown aetiology. His findings support those seen in the participants with Addison's disease.

4.4.6.2. Salivary androgens in CAH

Participants with CAH show a different salivary profile compared to what is seen in Addison's disease. Testosterone, A4, 11KT and 11OHA4 concentrations are all significantly higher than concentrations seen in matched controls. Again, the sample prior to the morning dose of hydrocortisone is not included. We hope to have these data in time. Serum testosterone and A4 are used routinely to manage CAH. Salivary sampling may be a reasonable alternative. Serum 11KT and 11OHA4 have been found to be higher in children and adults with CAH [137]. Serum and salivary testosterone, A4, 11KT and 11OHA4 are tightly correlated [128]. These data support that salivary concentrations of all four androgens are high in children and young people with CAH. Previous findings in adults suggest that 11KT is associated with increased

adrenal mass on CT in patients with CAH, and increased risk of testicular adrenal rests tumour (TART) in male adults with CAH [143]. It will be difficult to find appropriative comparatives in children with CAH. TARTs are rare in children. CT scans of the adrenal glands would be difficult to perform. It is also unsure whether the rise in 11KT is because of the increased adrenal mass or if the increased 11KT because of poor control leads to the increased adrenal mass. Growth parameters and bone age may be appropriate markers of good control. However, as can be seen in the GRACE 1 study, height SDS is within the normal range for the general population. It may be that pubertal growth is affected in CAH leading to the reported reduced final adult height that can be seen in CAH [179]. Growth may become a better marker of control during the peri-pubertal period. Many factors will need to be considered including clinical assessment of over and under treatment with glucocorticoids and comparisons made with salivary or serum concentrations of the 11oxC19 steroids. However, as can be seen in GRACE 1 there is also an increase in cardiovascular risk factors. These end points may prove useful when assessing 11oxC19 steroids as markers of 'good control', with an aim to improve morbidity and mortality into adulthood.

4.4.7. What is novel?

To my knowledge, higher mean glucose concentrations, measured using CGMS, have not been described in children and young people with primary AI. The primary outcome to assess for evidence of hypoglycaemia in this population does not seem to be the case. Although, one young person had evidence of hypoglycaemia for >2% of the time, this was not the general trend in the whole cohort studied. Cortisol is involved in glucose regulation. It may be that a non-physiological cortisol profile could lead to abnormalities in this control, leading to higher concentrations and therefore predisposing to the increased incidence of diabetes that has been described in the adult cohort. Although, as a whole cohort the diurnal profile seems to be that similar to physiological secretion, individual salivary profiles (see appendix 9.6) show peaks and troughs of salivary cortisol concentrations throughout the day.

No cardiovascular events were described in the study cohort, as we would expect given the age of participants. However, evidence of surrogate markers of cardiovascular and metabolic disease were common. Single risk factors may be found in some participants but often several risk factors were present in single participants. This supports previous literature. There are very little data regarding cardiovascular risk in children and young people with Addison's

disease. This is a rare disease, particularly in children, and therefore it is difficult to perform large studies in this cohort. We describe four participants with Addison's disease, aged 12 – 16 years, who had AI for 1 – 2 years. Cardiovascular risk factors are present in this cohort, even only several years after diagnosis.

FMD reduction has not been described previously in children and young people with primary AI. CIMT measurements have not been described in Addison's disease. These novel findings suggest that endothelial dysfunction may be present at a young age. Interventions aimed at improving cardiovascular health and maintaining endothelial function are required from the onset of diagnosis and should be addressed regularly with patients during clinical follow up.

4.4.8. Limitations

A power calculation was performed to estimate the number of patients required to identify a significant difference in the duration of hypoglycaemia in study patients compared to a cohort of healthy children [38]. If the assumption is made that the true proportion of those meeting criteria is 1% 16 participants were required to estimate proportion with 5% precision. If true proportion is 2% 31 participants were required. Primary AI is a rare disease. Only 44 patients were available to approach at Alder Hey Children's hospital, a tertiary endocrinology unit in the UK. 26 participants were enrolled. This was powered to detect hypoglycaemia (<3mmol/L) for >2% of the time. It was not powered for the secondary cardiovascular outcomes and this needs to be considered when interpreting results.

This was an observational study. Many results were compared against published reference ranges. Healthy controls, recruited from the local population would have been ideal to aid distinction between cardiovascular risk that may result from environmental factors and those that are likely to be attributed to AI and its treatment. However, it was thought that healthy children may not volunteer due to the intensity and nature of the study. Reference data were developed from populations that broadly mirrored the GRACE1 population and therefore, no healthy controls were recruited.

CGMS measures interstitial fluid rather than plasma glucose. There is good evidence that interstitial glucose correlates well with plasma glucose but with an approximate 15-minute delay. This should not affect data as percentage of time over 24 hours for seven days has been measured. CGMS may be inaccurate for the first 24 hours after insertion [159, 180, 181]. This

was evident on several glucose profiles, showing periods of hypoglycaemia after insertion, which then corrected for the next six days. In view of the documented inaccuracies the first 24 hours data were removed. The first 24 hours of the normative data were also removed to ensure a direct comparison.

Monitoring of glucose concentrations tend to be accurate with CGMS, particularly Dexcom G6®. This device is used in closed-loop technology for children and adults with type 1 diabetes mellitus. However, there can be times when readings may be inaccurate. For example, compression of the device can occasionally lead to false hypoglycaemic readings. One child showed evidence of spurious hyperglycaemia during CGM. CGMS is a trusted device. However, as with all technology the whole clinical picture needs to be considered. Reference glucose data also used a Dexcom G6® and therefore parameters should be comparable.

Ultrasound scanning was performed by the author. Training had taken place by a supervisor who was trained in both CIMT and FMD scanning. This author is not an ultra-sonographer. For both CIMT and FMD, training initially took place remotely, using media platforms. Laws in place because of the COVID-19 pandemic meant that face-to-face training was more limited than was initially expected. However, prior to the study opening I had made satisfactory progress and was deemed competent. Reproducibility data showed less than 10% coefficient of variability in five adults subjects. More than 50 CIMT and FMD studies had been performed prior to the study opening. Analysis training also took place remotely. CIMT analysis is automated using Q-lab in the ultrasound software. FMD analysis is semi-automated, using cardiovascular suite. Initial analysis was supported by the supervisory team during training.

4.4.9. Implications for clinical care

Whilst it was initially thought that hypoglycaemia may be a problem in children and young people with primary AI this does not seem to be the case. One participant did show asymptomatic hypoglycaemia on the background of a recent adrenal crisis with no obvious precipitant, and we therefore need to remain vigilant to clinical symptoms and signs on a case-by-case basis. However, generally within this population, evidence is limited that hypoglycaemia is an area for concern when well and on daily, maintenance hydrocortisone. No participant was unwell or on 'sick day' hydrocortisone during this study, therefore we cannot draw conclusions related to glucose parameters under these circumstances. It is assumed that all participants had good compliance with medication for the duration of the

study, which was documented on their exercise diaries and therefore we cannot describe blood glucose readings in the absence of medication.

Primary AI, of any cause, seems to be associated with an increased prevalence of cardiovascular risk factors. It is important that these risk factors are addressed in the clinical setting on a regular basis. It is recommended that children and young people with AI have their BP measured regularly [48, 94, 117]. This is particularly important in those children and young people taking fludrocortisone. Renin can be measured to guide dosing but it seems that BP, a non-invasive clinical measurement, correlates well with dose. BP should be taken, adjusted for age, sex and height and used to ensure that a child is not hypertensive. It seems that ABPM is not tolerated well in the younger population. Regular clinic BP measurements may be as useful in those it is not tolerated in. We cannot confirm this as those with high clinic BP were either too young or unable to tolerate ABPM. Recommended treatment of hypertension in adults with primary AI suggest a reduction in fludrocortisone dose. If this does not correct the hypertension, then fludrocortisone should not be stopped but an anti-hypertensive given instead [48].

CIMT and FMD are generally well tolerated. However, training is intense and it is used more widely in research rather than clinical settings. It is important to consider cardiovascular and metabolic risk factors as a whole. They should be addressed and treated. Height, weight and BMI measurements should be taken at each clinic appointment. Lifestyle advice should be given including a healthy, balanced diet; regular exercise and absolute avoidance of smoking. If there is a significant BMI increase, then additional support may need to be considered.

Hydrocortisone dosing should be maintained at levels to avoid under and over treatment. Hydrocortisone dosing was not high in this cohort. Formulations with a more physiological profile could be considered.

4.4.10. Recommendations for future research

This study was powered to assess hypoglycaemia using normative data. A cohort study with matched controls would be useful to ensure that the reference data was appropriate for the study population. A large, prospective study commencing in childhood and extending into adulthood would assess whether these cardiovascular risk factors mount to actual cardiac events in later life. Interventional studies, assessing reductions in hypertension, support for

weight management, nutritional advice and exercise programmes may improve cardiovascular risk over lifetime. Currently such services are becoming more available for those with complications of excess weight. Children with primary AI on hydrocortisone treatment may require intervention earlier than controls without.

New treatments are now available for AI. Efmody® has been licensed for use in those with CAH over 12 years of age. Future studies to assess the effect of the more physiological profile would be interesting, taking into consideration alterations in glucose metabolism, cardiovascular health and assessment of risk factors and associate them with salivary profiling of cortisol, cortisol and other adrenal biomarkers. Other medications such as CRF1 antagonists are also in early stages of development. The reduction in ACTH and therefore androgen production may also have an effect on cardiovascular health.

5. Chapter 5: Glucose regulation and cardiovascular health in children and young people with secondary AI (GRACE 2)

5.1. Introduction

In this chapter I will discuss a second study, in which glucose regulation, cardiovascular health and salivary cortisol and cortisone profiles are assessed in a cohort of children with secondary AI. Following the previous study, in which a higher mean glucose was evident, the primary outcome for this study was powered to review evidence of hyperglycaemia. Assessment of cardiovascular risk factors were undertaken to determine whether these risk factors are already present in childhood. Participants underwent assessment of BP, CIMT, FMD, and biochemical factors of their metabolic health. In contrast to the GRACE 1 study, salivary cortisol and cortisone samples were matched to hydrocortisone doses with the aim of reducing contamination in samples collected immediately prior to doses and describing the peaks and troughs in cortisol and cortisone during hydrocortisone therapy.

5.2. Methodology

5.2.1. Aims and objectives

- To describe glucose, BP and salivary cortisol and cortisone profiles, and biochemical and vascular markers of vascular health to:
    - Report the prevalence, frequency and severity of hyperglycaemia in the largest cohort of children and young people with AI studied to date, and its relationship with cortisol profiles.
    - Examine for associations between cortisol and cortisone profiles, BP disruption, markers of vascular endothelial dysfunction and biochemical determinants of cardiovascular risk to identify potential treatment targets.

5.2.2. Primary outcome

- Number of participants with glucose measurement >10mmol/L for more than 2% of the time [38].

5.2.3. Secondary outcomes

- Average systolic and diastolic clinic BP and, in those old enough, for a 24-hour period by use of ABPM [151].
- Number of participants with evidence of vascular dysfunction determined by measurement of CIMT and brachial artery FMD.

- Number of participants with insulin resistance defined by a rise in HOMA-IR for age and sex [152].
- AUC and mean salivary cortisol and cortisone throughout the day versus hydrocortisone dose (mg/m^2 body surface area/day).
- AUC and mean salivary cortisol and cortisone versus BP, mean glucose on CGMS and HOMA-IR.
- Hydrocortisone dose versus BP and mean glucose on CGMS and HOMA-IR.
- Salivary cortisol and cortisone concentration during periods of hyperglycaemia / hypotension / hypertension.

5.2.4. Inclusion criteria

- Age 2 years to 18 years.
- Structural abnormality of the hypothalamus or pituitary *or* cranial irradiation including the hypothalamic-pituitary axis in the field of irradiation.
- Secondary AI diagnosed on the low dose short Synacthen test (peak cortisol <350nmol/)[13] treated with daily hydrocortisone replacement therapy.
- Patients with additional pituitary hormones deficiency were eligible to participate if hormone replacement was satisfactory assessed by clinical evaluation and measurement of electrolytes, paired urine and serum osmolality, IGF-I, free thyroxine (fT4) and where appropriate oestrogen and testosterone.
- Written informed consent and where appropriate, assent.

5.2.5. Exclusion criteria

- Patients with additional diagnoses or treatment likely to influence blood glucose or BP.
- Patients aged <5yrs were excluded from studies of cardiovascular function.

5.2.6. Sample size

The power calculation was derived from an expected population incidence of hyperglycaemia of 0.1% [38]. The incidence of hyperglycaemia was estimated from the GRACE 1 data that were available at the time (following first 13 recruits). It was deemed that 20 participants would be required for 80% precision.

47 patients were found to be eligible for recruitment at Alder Hey Children's NHS foundation trust. 31 patients were approached at which point the recruitment target had been reached and recruitment stopped. One child was no longer eligible as a Libre Freestyle monitor had

been inserted due to clinical concern, two were needle phobic, two did not reply to correspondence and one was not brought on four occasions. Two patients were not approached as it was not deemed appropriate by their lead consultant, one was having their cortisol axis retested, one had no capacity for assent and one patient was busy with college work. 20 participants were recruited. A minimum of seven days was given between the time that the patient received information about the study and consent.

5.2.7. Study visit and differences in protocol

Study visits for GRACE 2 were set up to replicate the study visits for GRACE 1 (section 4.2.9). The main differences were:

(i) In the design of the GRACE 1 study, it was anticipated that paediatric patients would be at risk of hypoglycaemia, as discussed previously. For this reason, the primary outcome for GRACE 1 was hypoglycaemia (>2% of the time with a glucose <3mmol/L). Hyperglycaemia was seen more commonly than hypoglycaemia in GRACE 1. Given that long standing hyperglycaemia may have an adverse effect on cardiovascular health, and that patients with secondary AI may be at greater risk of hyperglycaemia due to disruption of other endocrine pathways, together with hypothalamic obesity, the primary outcome was changed to assess for hyperglycaemia (>10mmol/L for >2% of the time) for GRACE 2.

(ii) Past medical history included oncological treatment if appropriate.

(iii) Salivary sampling commenced at the study visit and was then timed prior to and two hours post hydrocortisone doses. Sampling continued until prior to the first dose on the following day to ensure early morning sampling. An additional diary was completed to ensure that times and date of sampling were recorded.

(iv) Biochemical measurements included cortisol, ACTH, insulin like growth factor 1 (IGF1), follicle stimulating hormone (FSH), luteinising hormone (LH), oestradiol or testosterone depending on sex, thyroid function tests, U&Es, HbA1c (if on growth hormone), fasting glucose, insulin, leptin, von Willebrand antigen and activity. Renin, aldosterone and metadrenalines were not measured.

5.2.8. Statistical considerations

Continuous data was subjected to the Shapiro-Wilk test to determine the nature of its distribution and expressed as mean (SD) or median (interquartile range), as appropriate.

Categorical variables are reported as counts with percentages. Continuous variables are compared using independent t-tests or Mann Whitney-U test, as appropriate. Categorical data are analysed using chi-squared test or Fishers exact test as appropriate. Correlations were performed using Spearman rank correlation analysis. Prevalence of the hypoglycaemia was calculated. Simple linear regression models were used to assess the relation between BP values, cortisol profiles, endothelial dysfunction and biochemical determinants of cardiovascular risk. Two-tailed P values <0.05 are considered statistically significant.

5.2.9. Ethics and governance

All aspects of the study were delivered according to the standards of Good Clinical Practice, and internal research governance standards. A paediatric endocrinologist reviewed participants' data. Clinically significant results were treated according to standard practice.

Ethical approval was issued by Queens Square (London) ethics committee prior to opening of the study in November 2021. (IRAS no: 312402 REC reference 21/PR/1163)

5.3. Results

5.3.1. Patient characteristics

Twenty participants (9 males) were recruited to GRACE 2. A summary table and individual participant data can be seen in appendices 9.10and 9.11. Patient characteristics are shown in Table 5.1. Six (30%) participants had congenital causes of secondary AI (five with septo-optic dysplasia and one with pituitary stalk interruption syndrome). Fourteen participants had acquired causes of secondary AI, including nine who had a craniopharyngioma, one with a 3rd ventricular/hypothalamic tumour, one had a medulloblastoma with craniospinal radiotherapy, one had a transitional meningioma with proton beam therapy, one had a hypothalamic glioma with neurofibromatosis type 1, and one with a primitive neuro-ectodermal tumour which had been treated with surgery and chemotherapy. 18/20 (90%) participants classed themselves as white British, 1 (5%) had 'other mixed' background, 1 (5%) was Black African. Pituitary axis function of the participants can be seen in Table 5.2. 3/20 (15%) participants were treated with metformin. 2/20 (10%) required treatment with melatonin. 2/20 (10%) were treated with anti-hypertensives. Each participant's results can be seen in a summary table in appendix 9.10 Details of each participant's glucose profile, cardiovascular risk factors and salivary profiles can be seen in appendix 9.11.

Table 5.1: Table showing characteristics of participants enrolled into GRACE 2

Parameter	Whole cohort	Congenital causes	Acquired causes
	N=20	N = 6	N = 14
	(i) Age and length of diagnosis (median, range)		
Age, years	13.0 (2.0 – 19.0)	10.0 (2.0 – 13.0)	14.5 (8.0 – 19.0)
Age at diagnosis of secondary AI, months	4.5 (0.0 – 16.0)	0.0 (0.0 – 0.0)	7.0 (3.0 – 16.0)
Length of time diagnosed with secondary AI, years	7.0 (1.0 – 13.0)	10.0 (2.0 – 13.0)	7.0 (1.0 – 11.0)
	(ii) Auxology (median, range)		
Height SDS	-0.2 (-2.2 – 1.9)	-0.3 (-2.2 – 1.9)	-0.4 (-1.9 – 1.9)
Weight SDS	1.8 (-1.9 – 8.4)	1.0 (-1.7 – 8.4)	2.2 (-1.9 – 5.8)
BMI SDS	2.1 (-2.3 – 3.9)	1.4 (-0.2 – 3.9)	2.3 (-2.3 – 3.4)
	(iii) Medication doses (median, range)		
Hydrocortisone dose, mg/m^2/day*	8.4 (6.8 – 11.8)	7.9 (6.8 – 10.9)	8.6 (7.1 – 11.8)
Sick day hydrocortisone dose (mg/m^2/day)	18.6 (14.3 – 28.6)	18.9 (14.3 – 21.3)	18.3 (14.3 – 28.6)
	(iv) Cardiovascular risk factors (N, %)		
1st degree relative with cardiovascular disease	1 (5%)	0 (0%)	1 (7%)
Co-existing familial hypercholesterolaemia	0 (0%)	0 (0%)	0 (0%)
1st degree relative with non-familial lipid abnormalities	0(0%)	0(0%)	0 (0%)
Parental smoking	7 (35%)	4 (66.7%)	3 (21.4%)
Participant smoking	0 (0%)	0 (0%)	0 (0%)

N: number, AI: adrenal insufficiency, SDS: standard deviation score, BMI: body mass index, mg: milligrams, %: percentage.

*One participant received modified release hydrocortisone, all other participants received standard formulation hydrocortisone

Table 5.2: Table showing TSH, GH, GnRH and ADH axis involvement in GRACE 2 participants

Hormonal axis affected	Number of participants requiring treatment for axis (%)	Medication and dose of those requiring treatment
TSH deficiency	18 (90)	Levothyroxine - 25-150 mcg/day
GH deficiency	18 (90)	Growth hormone - 20.9 ± 9.3 mcg/kg/day*
GnRH deficiency	8 (40) **	Oestrogen in females as patch for those in puberty and oral oestrogen for one who had completed puberty Testosterone injections for those in puberty. Testosterone gel for one who had completed puberty
Precocious puberty	1 (5)	No longer on GnRH analogue
ADH deficiency	10 (50)	Desmopressin***

TSH: thyroid stimulating hormone, GH: growth hormone, GnRH: gonadotrophin releasing hormone, ADH: anti-diuretic hormone, %: percentage, mcg: micrograms, kg: kilograms.
*GH doses include those on adult doses of GH **2 (10%) had progressed through puberty without assistance, a further 9 (45%) were too young for this to be assessed *** doses varied depending on clinical need

5.3.2. Glucose regulation

10/20 (50%) participants described symptoms of possible hypoglycaemia, usually related to mild illness or prior to the diagnosis of AI. Median fasting plasma glucose, measured after an overnight fast and after the morning dose of hydrocortisone, was 4.6mmol/L (range 3.6 – 5.6) Mean glucose and co-efficient of variation measured by CGMS over seven days can be seen in Figure 5-1. Mean glucose concentrations were higher in GRACE 2 participants 5.9 ± 0.4 (4.9 – 6.9) compared to healthy controls 5.5 ± 0.4 (4.9 – 5.9), $p<0.01$. Data from the reference population showed an increased co-efficient of variation of glucose measurements, but this was not significant (GRACE 2: 14.8 ± 2.6 (10.9 – 20.6) compared to healthy controls: 15.3 ± 2.8 (11.9 – 18.8), $p=0.05$). The percentage of time spent at <3mmol/L, <3.33, <3.39, 3.39 – 7.78, >7.78, >8.89 and >10mmol/L measured by CGMS over seven days can be found in Table 5.3. The first 24 hours have been excluded in both sets of data, to reduce the risk of inaccurate glucose measurements that can be seen within the first 24 hours of insertion. (See section 4.3.2.1 for further information).

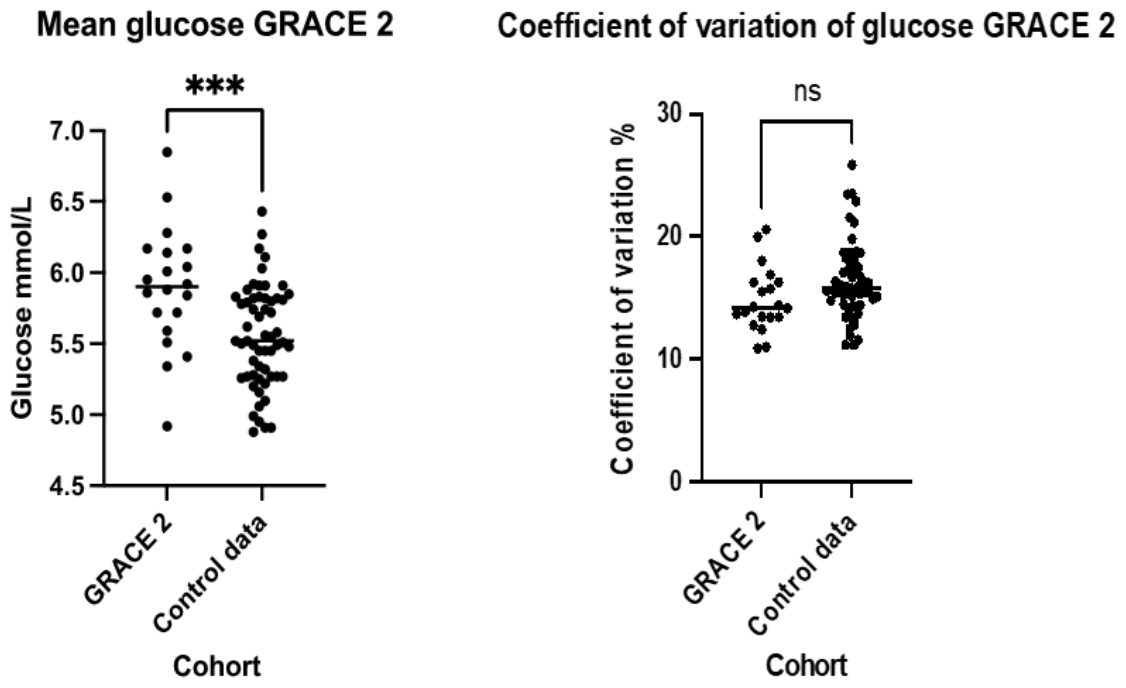


Figure 5-1: Figure showing mean glucose concentrations and co-efficient of variation between participants in GRACE 2 compared to healthy, controls

Table 5.3: Table showing difference in glucose results across the groups when comparing GRACE 2 data versus healthy, normative data.

	GRACE 2 participants	Control subjects	*P*-value before Bonferroni correction	Significance after Bonferroni correction
P(i)	**Percentage of time spent with glucose readings in each category, median (IQR)**			
>10.00 mmol/L	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.1)	0.14	NS
>8.89 mmol/L	0.2 (0.1 – 0.2)	0.0 (0.0 – 0.5)	0.67	NS
>7.78 mmol/L	3.0 (1.5 – 4.5)	1.1 (0.4 – 2.3)	0.19	NS
3.39 – 7.78 mmol/L	95.6 (92.3 – 97.4)	96.8 (91.9 – 98.7)	0.65	NS
<3.39 mmol/L	0.2 (0.01 – 1.4)	1.4 (0.5 – 3.7)	0.01[a]	NS
<3.33 mmol/L	0.1 (0.00 – 0.5)	0.3 (0.0 – 0.5)	0.74	NS
<3.00 mmol/L	0.0 (0.0 – 0.1)	0.1 (0.0 – 0.4)	0.83	NS

Mmol/L: millimoles per litre, SD: standard deviation, IQR: interquartile range, NS: not significant.
a – Significant result.

Mean glucose concentrations on CGMS correlated with hydrocortisone dose but not significantly so (r^2 = -0.41 95CI: -0.73 – 0.06, p = 0.076) (Figure 5-2). Interestingly, the mean glucose concentrations were lower in those with higher doses of hydrocortisone. Reflecting on these data it was hypothesised that those with a higher BMI may be on lower doses of hydrocortisone based on their body surface area. Using simple linear regression, this is shown

to be the case with r^2=0.27, p=0.02 (Figure 5-3). Mean glucose on CGMS does not correlate with age (r^2= 0.14, p=0.56) or with BMI (r^2= -0.29, p=0.23) (Figure 5-4).

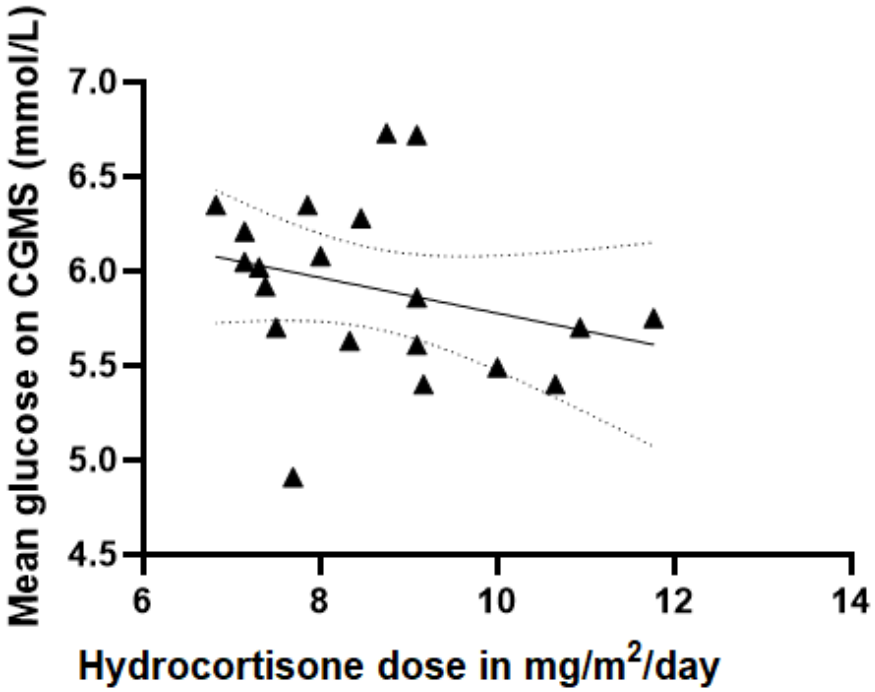


Figure 5-2: Figure showing mean glucose concentrations (mmol/L) on CGMS compared to hydrocortisone dose (mg/m²/day)

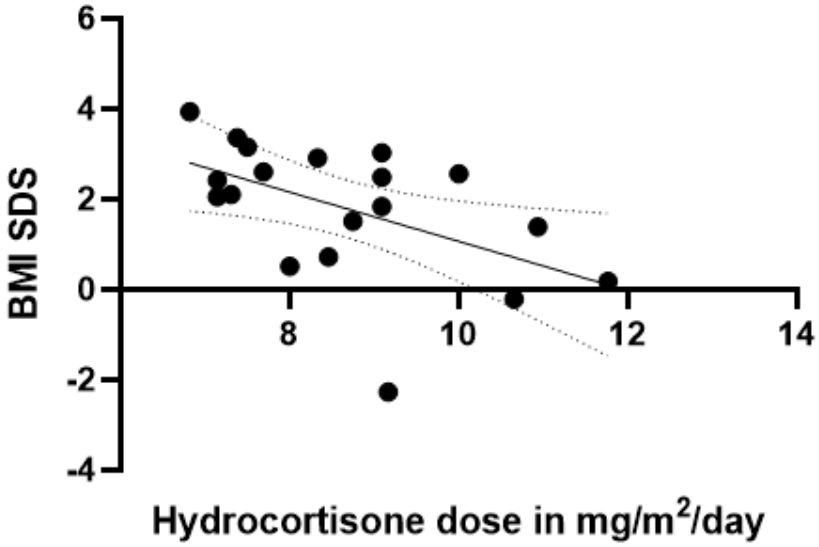


Figure 5-3: Graph showing hydrocortisone dose (mg/m²/day) compared to BMI SDS

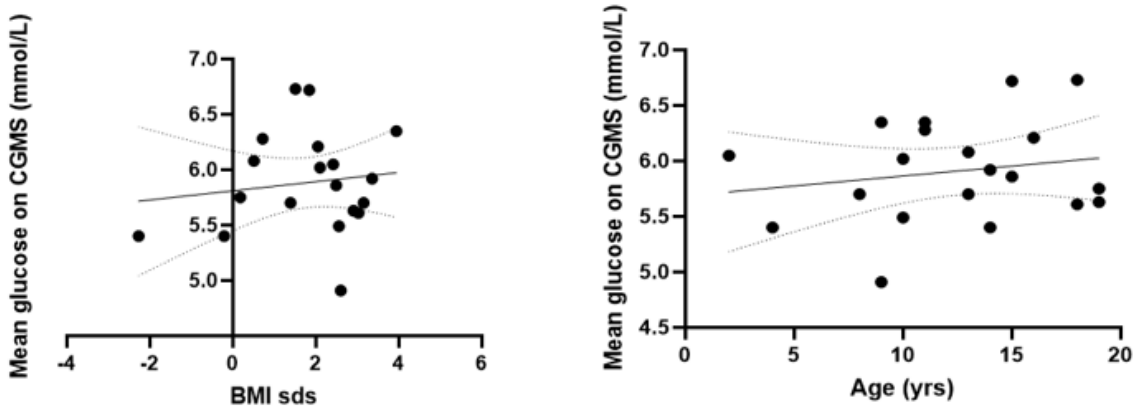


Figure 5-4: Mean glucose concentrations (mmol/L) on CGMS compared to BMI SDS and age (years)

5.3.2.1. Comparison to primary outcome

No child had blood glucose readings >10mmol/L for >2% of the time. Once data from the first 24 hours was removed, no child had interstitial glucose levels <3mmol/L for >2% of the time.

5.3.3. Cardiovascular health

5.3.3.1. Characteristics of participants eligible for assessment of cardiovascular function (>5 years)

Clinic BP was performed in all participants. Five participants were deemed unsuitable for ABPM as they were ten years or below and a clinical decision was taken that it would not be tolerated. Two participants, aged two and four years, both with septo-optic dysplasia were excluded from vascular scanning, based on their age. Venepuncture was attempted in all participants but was unsuccessful in two.

5.3.3.2. Clinic BP

Median, IQR and range can be seen in Table 5.4. Participants on anti-hypertensives have been excluded from this data. One participant on anti-hypertensives had a raised diastolic BP at the study visit. The other participant had a normal BP. BP across the whole cohort did not correlate with hydrocortisone dose.

Table 5.4: Table showing median, IQR and range for GRACE 2 population

Parameter	Median, IQR	Blood pressure centile
Systolic BP percentile	71.0 (44.0 – 82.0)	36^{th}-96^{th}
Diastolic BP percentile	75.0 (46.0 –82.0)	9^{th} – 99^{th}

BP: blood pressure, IQR: interquartile range.

One participant had a clinic BP >95th centile, whilst 2 participants had a diastolic BP >95th centile. No participants had both a raised systolic and diastolic BP. Participants with a raised BP are shown in Table 5.5.

Table 5.5: Table showing participants with raised BP >95th centile

Participant	Age (years)	Sex (M/F)	Diagnosis	BMI SDS	Hydrocortisone dose (mg/m^2/day)
(i)	**Raised systolic BP only**				
213	14	F	Craniopharyngioma	3.36	9.4
(ii)	**Raised diastolic BP only**				
203	15	M	Third ventricular/hypothalamic tumour	2.49	9.1
209	19	F	Craniopharyngioma	0.18	11.8

M: male, F: female, BMI: body mass index, SDS: standard deviation score, mg: milligrams, BP: blood pressure.

5.3.3.3. Ambulatory BP monitoring (ABPM)

15/20 (75%) were fitted with an ABPM. The median (IQR) percentage of successful readings was 75% (55.7 – 82.7%). One participant did not tolerate the BP cuff overnight.

All ABPM systolic BPs were <95th centile. One ABPM diastolic BP average was on the 95th centile, all others were <95th centile. The participant who had a diastolic BP on the 95th centile was an 18-year-old male with a BMI SDS of 3.03 and hydrocortisone dose of 9.1mg/m^2/day (20mg/day). Loss of systolic nocturnal dipping was evident in 8/14 (57%) participants. Loss of diastolic nocturnal dipping was evident in 5/14 (36%) participants. Participants on anti-hypertensives have been excluded from Table 5.6, which shows clinic BP and ABPM results. The two participants on anti-hypertensives had average systolic BP measurements <50th centile for age and sex. One participant had a diastolic BP <50th centile, whilst the other had a diastolic BP<75th centile.

Table 5.6: Table showing the number of participants who had a BP >90th centile on clinic BP and ABPM

		BP centile, n (%)		
		<90th	**≥90th**	**≥95th**
I.	**Clinic BP for age, gender and height (n=18)**			

Systolic	16 (88.9)	2 (11.1)	1 (5.6)
Diastolic	1 (94.4)	1 (5.6)	1 (5.6)
II.	**ABPM for age and gender (n=13)**		
Overall systolic	8 (61.5)	5 (38.4)	0 (0)
Overall diastolic	9 (69.2)	3 (23.1)	1(7.7)

BP: blood pressure, n: number.

5.3.3.4. Carotid intima media thickness (CIMT)

CIMT was performed in 18/20 (90%) participants. 4/18 (22.2%) had CIMT measurements >95th centile and a further one participant who had a measurement on the 90th centile. The number of participants with CIMT measurements on each percentile can be seen in Table 5.7.

Table 5.7: Table showing CIMT percentiles in GRACE 2 population compared to normative data

Percentile of CIMT measurement based on age and sex	**Number of participants with CIMT measurement within percentile category (n = 18)**
50th-75th	4 (22.2%)
75th-90th	8 (44.4%)
90th-95th	2 (11.1%)
>95th	4 (22.2%)

CIMT: carotid intima-media thickness, n: number.

Those participants with CIMT measurements >95th centile for sex and age can be seen in Table 5.8.

Table 5.8: Features of participants with CIMT percentiles >95th centile for age and sex

Participant	Age (years)	Sex (M/F)	Diagnosis	BMI SDS	SBP percentile	DBP percentile	Hydrocortisone dose ($mg/m^2/day$)	Other pituitary axes affected
201	16	F	Craniopharyngioma	2.1	71	46	7.1	TSH, GH, GnRH, ADH
215	18	M	Hypothalamic glioma, NF1	1.5	71	24	8.8	TSH, GH, PP, ADH
217	13	F	Septo-optic dysplasia	1.39	44	56	10.9	TSH, GH, GnRH
218	15	M	Septo-optic dysplasia	1.84	67	13	9.1	GH

M: male, F: female, BMI: body mass index, SDS: standard deviation score, SBP: systolic blood pressure, DBP: diastolic blood pressure, mg: milligrams, TSH: thyroid stimulating hormone, GH: growth hormone, GnRH: gonadotrophin releasing hormone, ADH: anti-diuretic hormone, PP: precocious puberty, NF1: neurofibromatosis type 1.

5.3.3.5. *Flow mediated dilatation*

18/20 (90%) participants were eligible for FMD measurement as they were aged 5 years and over. One child had taken their anti-hypertensives on the morning of the study and therefore was excluded. A further participant had artefact on the study recording and therefore the study could not be analysed. One participant could not tolerate the procedure. The results of the remaining 15 measurements can be seen in Table 5.9. FMD measurements were below 7% in 3 (20%) participants. Features of participants with low FMD can be seen in Table 5.10. Normal FMD measurements are between 7 – 15%. The other parameters in this table are given for information only.

Table 5.9: Table showing results of FMD measurement in GRACE 2

Measurement on ultrasound	Median (IQR)
FMD (%)	8.6 (6.7 – 15.1)
Baseline diameter (mm)	3.2 (2.9 – 4.1)
Maximum diameter (mm)	3.5 (3.1 – 4.5)
Time to maximum diameter (seconds)	404.4 (392.2 – 435.1)

FMD: flow mediated dilatation, mm: millimetres, IQR: interquartile range.

Table 5.10: Table showing participants with an FMD value <7%

Participant	***Age (years)***	***Sex (M/F)***	***Diagnosis***	***BMI SDS***	***SBP percentile***	***DBP percentile***	***Hydrocortisone dose (mg/m^2/day)***	***CIMT centile***
212	8	M	Craniopharyngioma	3.15	38	44	7.5	75th-90th
214	9	M	Craniopharyngioma	2.6	67	76	7.7	75th-90th
215	18	M	Hypothalamic glioma, NF1	1.51	71	24	8.8	>95th

M: male, F: female, BMI: body mass index, SDS: standard deviation score, SBP: systolic blood pressure, DBP: diastolic blood pressure, mg: milligrams, CIMT: carotid intima-media thickness, NF1: neurofibromatosis type 1.

5.3.4. Metabolic health

5.3.4.1. HOMA-IR

The median (IQR) HOMA-IR was 2.6 (0.8-6.0). HOMA-IR results by centile can be seen in Table 5.11.

Table 5.11: Table showing number of participants with a HOMA-IR result within each percentile category

Percentile of HOMA-IR measurement based on age and sex	Number of participants with HOMA-IR measurement within percentile category (n = 18)
<3rd	2 (11.1%)
3-10th	3 (16.7%)
10-25th	1 (5.6%)
25th-50th	1 (5.6%)
50th-75th	0 (0%)
75th-90th	1 (5.6%)
90-97th	3 (16.7%)
>97th	7 (38.9%)

HOMA-IR: homeostatic model assessment for insulin resistance, n: number.

HOMA-IR of those participants with a HOMA-IR >97th centile, along with the reference range can be seen in Table 5.12. Insulin resistance seems to be a significant concern and can be excessively high within this population. Of the three participants on metformin, one (study ID 201) had a raised HOMA-IR. The other two participants had a normal HOMA-IR.

Table 5.12: Table showing HOMA-IR for those with a level >97th centile

Study ID	Fasting glucose (mmol/L)	Insulin (pmol/L)	Calculated HOMA-IR	Reference range for age and sex
201	4.6	348	10.24	0.57 – 2.95
203	5.4	637	22.01	0.47 – 2.78
212	4.8	100	3.07	0.39 – 2.18
213	5.6	370	13.26	0.81 – 3.39
214	4.7	486	14.60	0.43 – 2.31
215	5.1	179	5.84	0.70 – 2.69
217	4.9	193	6.05	0.68 – 3.39

mmol/L: millimoles per litre, pmol/L: picomoles per litre, HOMA-IR: homeostatic model assessment for insulin resistance.

In those participants with a raised HOMA-IR, each had at least one other cardiovascular risk factor, which can be seen in Table 5.13. HOMA-IR did not correlate with hydrocortisone dose in mg/m^2/day.

Table 5.13: Table showing features of those participants in GRACE 2 who have a HOMA-IR >97th centile for age and sex

Participant	Age (years)	Sex (M/F)	Diagnosis	BMI SDS	SBP percentile	DBP percentile	Evidence of non-dipping on ABPM	Hydrocortisone dose (mg/m^2/day)	CIMT centile	FMD measurement (%)
201	16	F	Craniopharyngioma	**2.05**	71	46	**Yes**	7.1	**>95th**	18.80
203	15	M	Third ventricular/ hypothalamic tumour	**2.49**	89	**97**	No	9.1	50th-75th	**6.63**
212	8	M	Craniopharyngioma	**3.15**	38	44	**Yes**	7.5	75th-90th	**3.42**
213	14	F	Craniopharyngioma	**3.36**	**96**	88	**Yes**	7.4	75th	**6.69**
214	9	M	Craniopharyngioma	**2.6**	67	76	**Yes**	7.7	75th	**2.48**
215	18	M	Hypothalamic glioma, NF1	1.51	71	24	No	8.8	**>95th**	**4.47**
217	13	F	Septo-optic dysplasia	1.39	44	56	No	11.0	**>95th**	**6.75**

M: male, F: female, BMI: body mass index, SDS: standard deviation score, SBP: systolic blood pressure, DBP: diastolic blood pressure, ABPM: ambulatory blood pressure monitoring, mg: milligrams, CIMT: carotid intima-media thickness, FMD: flow mediated dilatation, NF1: neurofibromatosis type 1.

5.3.4.2. *Plasma leptin concentrations*

Plasma leptin concentrations have not yet been analysed due to staff shortages at the receiving laboratory.

5.3.4.3. *Other biochemical variables*

Fasting glucose, HbA1c and lipid profiles results can be seen in Table 5.14. No participant showed evidence of a low or elevated fasting blood glucose. All participants had a HbA1c within the normal range. Dyslipidaemia could be seen in some participants. No participant had evidence of raised VWF antigen or activity levels (Table 5.14).

Table 5.14: Table showing fasting glucose, HbA1c, lipid profiles and VWF antigen and activity in GRACE 2 participants

Biochemical variables	**Mean ± SD (range), mmol/L**	**Reference range, mmol/L**	**Frequency of low readings, n (%)**	**Frequency of high readings, n (%)**
Fasting glucose	4.6 ± 0.5 (3.6 – 5.6)	2.60 – 6.10	-	-
HbA1c	32.2 ± 2.4 (28.0 - 36.0)	20.00 – 42.00	-	-
Total cholesterol	4.5 ± 1.2 (2.9 – 7.7)	3.10 – 5.40	1 (5.6)	2 (11.1)
LDL-C	2.4 ± 0.9 (1.1 – 4.6)	<2.85	-	3 (16.7)
HDL-C	1.4 ± 0.5 (0.8 – 2.7)	>1.17	13 (72.2)	-
TG	1.6 ± 1.6 (0.4 – 5.4)	≥2.00		4 (14.3)
VWF antigen	117.5 ± 54.7 (56.4 – 226.6)	55 – 145	4 (14.3)	
VWF activity	111.1 ± 41.5 (58.0 – 183.1)	55 – 145	4 (14.3)	

SD: standard deviation, mmol/L: millimoles per litre, n: number, %: percentage, LDL-C: low-density lipoprotein cholesterol, HLD-C: high-density lipoprotein cholesterol, TG: triglycerides, VWF: von Willebrand's factor.

5.3.5. Salivary cortisol and cortisone

Salivary cortisol and cortisone measurements were collected by all 20 participants and profiles can be seen in appendix 9.11. They provided a sample at the study visit one to two hours post their morning dose of hydrocortisone. Participants then took further samples prior to each dose and two hours post each dose. The final sample was taken on the following morning prior to the first dose. 118 samples were collected and four

were insufficient. 10/20 (50%) of participants had been woken on the morning of the study.

5.3.5.1. Salivary cortisol

Figure 5-5 shows salivary cortisol concentrations (nmol/L) in participants with secondary AI, treated with hydrocortisone compared to healthy children (Chapter 3). A significant difference, p=0.0012 was seen between the groups. As found in chapter 4, there was evidence of hydrocortisone contamination of saliva samples and salivary cortisol measurements greater than 78nmol/L (twice the median of the peak seen in healthy children) were removed (n=19) from the analysis. Data including the likely contaminated samples are shown in Figure 5-5, and following the removal of samples likely to be contaminated, in Figure 5-6. The difference between the groups remained significant after contaminated samples were removed with a p value of 0.004.

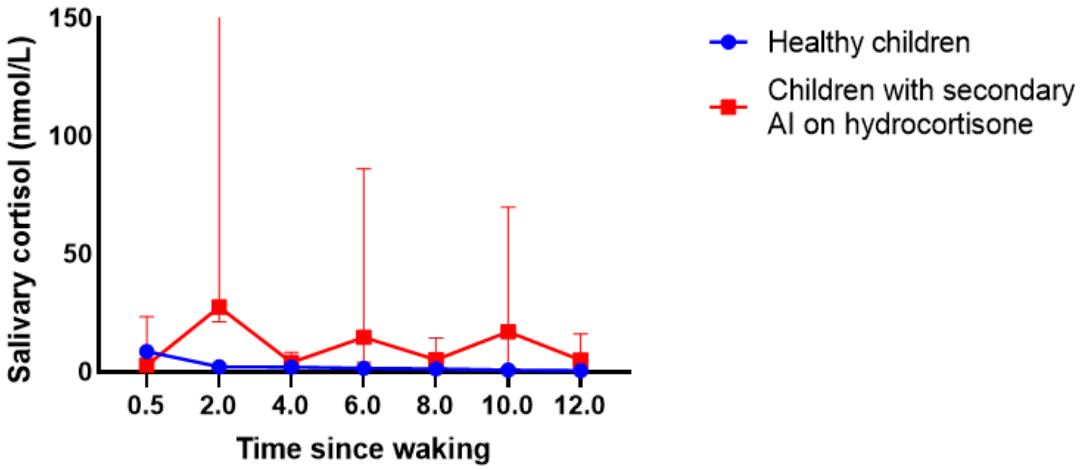


Figure 5-5: Graph showing salivary cortisol (nmol/L) in participants with secondary AI treated with hydrocortisone compared to healthy children.

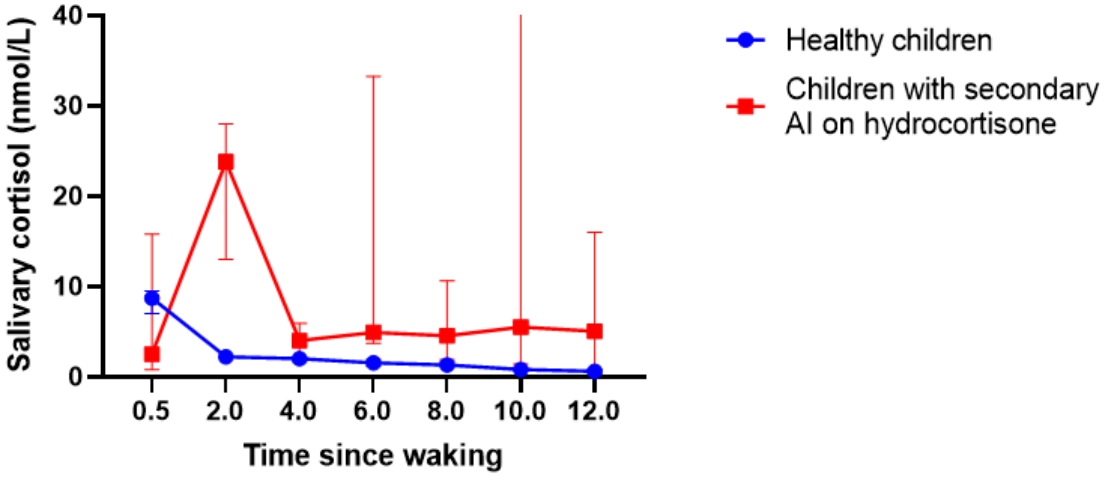


Figure 5-6: Graph showing salivary cortisol (nmol/L) in participants with secondary AI treated with hydrocortisone compared to healthy children (likely contaminants removed).

5.3.5.2. Salivary cortisone

Salivary cortisone measurements can be seen in Figure 5-7. Although the salivary cortisone pattern does not show a diurnal profile, there is no difference between median and 95% CI between the groups throughout the day, p=>0.999 using the Mann Whitney U test suggesting a similar total cortisol exposure. However, there are peaks and troughs of salivary cortisone pre and post dose that do not reflect the normal diurnal rhythm seen in healthy children and young people.

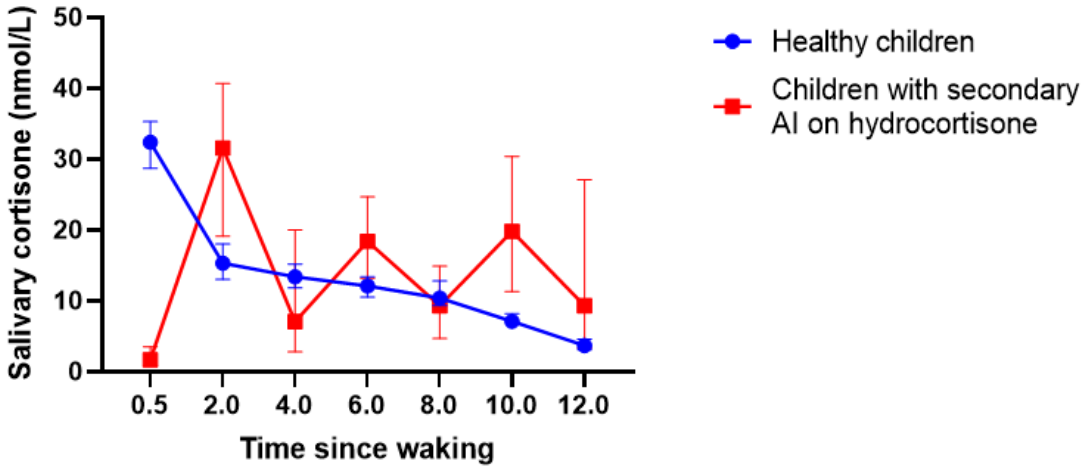


Figure 5-7: Graph showing salivary cortisone (nmol/L) in participants with secondary AI treated with hydrocortisone compared to healthy children.

Mean salivary cortisone does not significantly correlate with hydrocortisone dose (r^2= 0.38, 95%CI: -0.09 – 0.71, p= 0.09) but a trend can be seen.

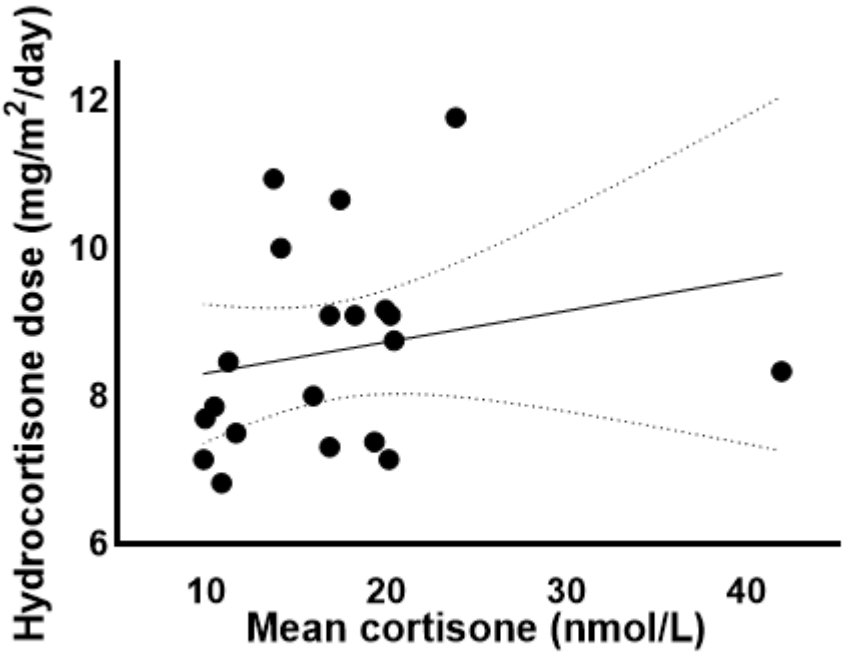


Figure 5-8: Graph showing mean cortisone (nmol/L) throughout the day compared to hydrocortisone dose (mg/m²/day).

Salivary cortisone concentrations could not be correlated with periods of hyperglycaemia as salivary sampling occurred on the first day of the study. Glucose data were excluded during this time period. Mean salivary cortisone did not correlate with BP, mean glucose on CGMS over seven days or HOMA-IR.

5.4. Discussion

This study has contributed the first dataset assessing glucose concentration via CGM over a one-week period in children and young people with secondary AI, showing higher mean glucose concentrations compared to a population of healthy children. Prolonged periods of hypoglycaemia have not been shown. Secondary outcomes show that children and young people have evidence of cardiovascular risk factors such as obesity, hypertension, increased CIMT, reduced FMD and raised biochemical markers of cardiovascular and metabolic risk. These are often clustered within the same participants, whilst some have no evidence of increased cardiovascular risk. A summary table can be seen in appendix 9.10. Salivary cortisol sampling is likely contaminated by oral hydrocortisone. Salivary cortisone shows a similar total cortisol exposure between those treated with hydrocortisone and healthy children, however a diurnal rhythm is not seen. Instead, peaks and troughs in salivary cortisone concentrations occur pre and post hydrocortisone dosing.

5.4.1. Glucose

Mean glucose concentrations are higher in the population with secondary AI when compared to healthy children [38]. However, there seems to be no difference in variability of glucose concentration shown by measuring the co-efficient of variation. Prior to a Bonferroni correction it seemed that healthy children were more likely to show evidence of hypoglycaemia (<3.9mmol/L) than compared to the population of children with secondary AI, on hydrocortisone. However, once this was corrected for the number of tests performed, this difference was no longer significant. There was no difference between interstitial fluid glucose readings <3mmol/L between the two populations. Each dataset was treated identically as the raw data were obtained for the healthy control cohort [38]. Both datasets had the first 24 hours removed to ensure consistency in interpretation and accounting for inaccuracies that have been shown to occur during this period [159, 160].

Hypoglycaemia has been described in children who have both AI and GH deficiency when medications are omitted [66]. This study has shown that children and young people who have secondary AI, whilst well, taking their hydrocortisone treatment and all other treatments required for other pituitary axis deficiencies, show no evidence

of hypoglycaemia. Hyperglycaemia (glucose concentrations greater than 10mmol/L for greater than 2% of the time) has not been shown in GRACE 2. Higher mean glucose concentrations of 5.9 ± 0.4mmol/L compared to 5.5 ± 0.4mmol/L can be seen within the GRACE 2 cohort. Although this shows a statistically significant difference, the clinical significance of this increase in glucose concentrations is unknown. Previous studies assessing glucose concentrations in healthy adults suggest that even borderline raised glucose concentrations (>7.78mmol/L) can have an impact on cardiovascular risk [167]. Mean higher glucose concentrations for many years may exacerbate the increased cardiovascular risk that has been shown in participants within this cohort.

Interestingly, this cohort, although not statistically significant showed a trend towards lower glucose concentrations at higher doses of hydrocortisone. Further explanation regarding this was sought. A possible hypothesis was that those with a higher BMI are on lower doses of hydrocortisone for their body surface area. A significant correlation between BMI SDS and hydrocortisone dose can be seen. It is possible that clinicians are mindful to use the lowest possible dose of hydrocortisone, that allows children to be symptom free, particularly on those with higher BMI, to avoid an excessive weight gain with higher doses of hydrocortisone. Mean glucose increased slightly for BMI and age, however these findings were also not statistically significant. It would be interesting to see if these findings are consistent in a larger cohort.

5.4.2. Cardiovascular health

Clinic BP can be assessed manually or with semi-automated devices [161]. Hypertension in adults has been shown to be associated with increased risk of adverse cardiovascular events [182]. Cardiac events are rare in children therefore surrogate markers are used as an alternative. These predictive markers are also used in adult study populations. Within this cohort, one participant had systolic BP >95th centile, whilst two participants had evidence of raised diastolic BP > 95th centile. Clinic BP may also be elevated from 'white coat hypertension'. Therefore, it is recommended BP is measured regularly. The clinic BP was measured at the beginning of the study visit. All children will have ongoing BP monitoring at clinic visits.

ABPM was performed in those aged 10 years and over. It is reassuring that no child had an average systolic BP >95th centile on ABPM. However, one child had a diastolic BP on the 95th centile, who will have ongoing monitoring of clinic BP measurements and be referred for further assessment if required. Loss of systolic nocturnal dipping (<10% fall) was evident in 8/14 (57%) participants and diastolic nocturnal dipping (<10% fall) was evident in 5/14 (36%) participants. Loss of nocturnal dipping is thought to be an early indicator of cardiovascular disease in normotensive and hypertensive adults [183]. Loss of nocturnal dipping has also been associated with raised CIMT and endothelial dysfunction in 9 – 19-year olds with systemic lupus erythematosus with paediatric onset [184]. High levels of non-dipping can be seen in the GRACE 2 cohort.

ABPM can be difficult to tolerate. This cohort was older than the cohort in GRACE1. Therefore, more participants were given the device. Only one participant did not tolerate it overnight, although, some participants did express discomfort the following day.

CIMT measurements were generally well tolerated in this cohort. Four participants had evidence of CIMT >95th centile with all participants having CIMT >50th centile. As discussed in section 4.4.3, ideally, we would compare CIMT measurements against healthy controls within a local population. However, this was not available, therefore we compared against reference ranges that were thought to be the most suitable. All participants with a raised CIMT had a BMI SDS >1.3, were normotensive and were treated with hydrocortisone doses between 7.1 – 10.9mg/m^2/day.

FMD was tolerated in 18 participants. However, the procedure and analysis were more difficult than CIMT measurements. FMD can be performed on those who have taken their anti-hypertensives on the morning of the study, however, this may impact the results [185], therefore one participant was excluded. Analysable data were available in 75% of the cohort. Low FMD measurements show evidence of endothelial dysfunction. Three participants had FMD readings below the normal reference range of 7%, all of whom had acquired secondary AI, had a BMI SDS of >1.5, were normotensive, were treated with hydrocortisone doses 7.5 – 8.8mg/m^2/day and had CIMT >75th centile. Combined cardiovascular risk factors of increased BMI and increased CIMT are evident in these participants. These participants require healthy

lifestyle advice, weight management guidance and close monitoring of their BP, with treatment if necessary.

5.4.3. Metabolic health

HOMA-IR was raised in 7 (36.8%) participants. Values were three to eight times the upper limit of the reference range for age and sex indicating that insulin resistance was common and could be severe. Of concern, these participants all have a BMI SDS >1.35, one has a clinic BP >95th centile, one has a clinic diastolic BP >95th centile, four have evidence of non-dipping on ABPM, three have CIMT >95th centile and three have evidence of endothelial dysfunction with FMD measurements less than 7%. Clustering of cardiovascular risk factors within participants is apparent. These children and young people warrant close monitoring and healthy lifestyle advice, whilst we await further interventions that may help to reduce cardiovascular risk. Raised triglycerides, high LDL-C, low HDL-C and high VWF antigen and activity are also thought to be associated with increased cardiovascular disease risk and can be seen within this population.

5.4.4. Salivary cortisol and cortisone

Measurements of salivary cortisol concentrations are likely to be of clinical value in patients treated with hydrocortisone due to the frequent drug contamination. This may be secondary to the dose remaining within the mouth, may be secondary to small cuts within the mouth which will mean that plasma cortisol will be measured rather than salivary. This reflects findings within previous literature [186]. The threshold that we set for the exclusion of samples in the analysis of cortisol >78nmol/L was arbitrary, and it is possible that in doing so we have under or over estimated total cortisol exposure. Recognising this possibility, the concentration of cortisol in the saliva of GRACE 2 participants was significantly higher than in healthy controls.

Salivary cortisol was low prior to the morning dose. This is the sample least likely to be contaminated as the previous hydrocortisone dose was taken the night before. However, high variability remained with 95th percentile being much higher than the controls at that time suggesting that not all samples were prior to this first dose or completely free of contamination.

Salivary cortisone seems to be less affected by contaminants. Overall salivary cortisone was similar to salivary cortisone concentrations seen throughout the day.

Salivary cortisone correlated strongly with plasma cortisol, and the data described in this study suggest that cortisol exposure throughout the day is similar in both groups. However, what is evident is the profile of salivary cortisone differs to that of healthy children. Earlier in the day and later in the evening peaks and troughs do not have 95th confidence intervals that overlap. This altered profile has been reported previously in measurements made in serum [187]. It is this altered profile that has been thought to be a contributing factor for cardiovascular risk [12].

5.4.5. What is novel?

This is the first description of glucose concentrations measured via CGMS over a seven-day period in patients with secondary AI, on maintenance therapy, and the first to report a generalised increase in glucose concentrations, without hyperglycaemia (>10mmol/L) or glucose variability. Cortisol has an important role in glucose homeostasis and it is possible that disruption in the circadian profile of cortisol exposure has an effect on glucose regulation in these study participants. This generalised increase in glucose concentrations may be the precedent to the increased prevalence of type 2 diabetes seen in later life in secondary AI [69, 188, 189]. Children with secondary AI seem to have a higher BMI than the general population which may also be a contributing factor.

Increased cardiovascular risk has been described previously in adults with secondary AI [120]. In this study population, cardiovascular risk factors are present and cluster within participants. Much larger studies would be required to investigate this clustering further. These data replicate previous findings showing an increased incidence of obesity. CIMT and FMD measurements, to my knowledge, have not been reported in children and young people with secondary AI. These findings provide evidence that endothelial dysfunction is present from childhood in these patients and interventions to improve cardiovascular morbidity and mortality should start in childhood.

5.4.6. Limitations

This is a study of a small and highly heterogeneous group of patients, many of whom have multiple co-morbidities. Congenital secondary AI is associated with global developmental delay, reduced mobility secondary to this, visual difficulties and

hypothalamic obesity. In paediatric practice, acquired secondary AI is usually caused by the direct effect of brain tumours, or their treatment. Each of these associations may also lead to increased cardiovascular risk. Glucose metabolism may also be affected by chemotherapy agents, and it would be difficult to unpick these associations and decipher which had the greatest impact [190, 191]. Patients with secondary AI usually have multiple hypothalamic-pituitary hormone deficiencies, and treatment with human GH (hGH), sex hormones and thyroxine may also influence cardiovascular and metabolic health. Participants were only eligible to take part in the study if they were on adequate hormone replacement. However, hGH administered as a daily injection does not mimic the physiological profile of GH secretion in healthy individuals. This is also true of treatment with once daily levothyroxine and oestrogen and testosterone, which are administered as patches, tablets, injections or gel, all of which differ in their bioavailability and pharmacokinetic properties.

In this small and heterogeneous population, it is not possible to determine which of the multiple risk factors have the greatest influence on the cardiovascular health, and the contribution of non-physiological cortisol is difficult to dissociate from other risk factors [192]. Newer MR-HC preparations may reduce the cardiovascular risk. Future studies comparing those on standard hydrocortisone formulations compared to modified release should be considered to address if reduced cardiovascular risk factors can be seen, both in adults and children. In truth, these findings show that we need to pay even closer attention to cardiovascular risk factors, no matter the cause, with an aim to reduce morbidity and mortality further as this population transitions into adulthood.

The study was powered to determine whether hyperglycaemic occurred more frequently in patients with secondary AI than in a healthy population (glucose >10mmol/L for more than 2% of the time). This power calculation was performed using data from healthy children [38] and preliminary data from GRACE 1, after the first thirteen participants. At the end of recruitment of the 26 participants of GRACE 1 the evidence of hyperglycaemia was not significant. This may have affected the power calculation. However, these were the best available data at the time of the power calculation.

5.4.7. Implications for clinical care

The slightly higher glucose measurements do not require acute intervention in this age group, over and above lifestyle advice. Although hypoglycaemia (<3mmol/L for 2% of the time) was not evident in this population, who were well at the time of the study, patients should still be counselled regarding symptoms and signs of hypoglycaemia as this remains a concern during adrenal crises. Families will not need counselling for signs and symptoms associated with diabetes or high blood glucose concentrations, aside from what is recommended for the general population.

Monitoring of weight, BMI and BP must be performed routinely. These are modifiable risk factors that can be addressed to reduce cardiovascular risk. Although, CIMT and FMD are measurements that are only used in research these data suggest that there is evidence of early cardiovascular dysfunction within the population therefore discussions regarding healthy lifestyle, diet and weight management should also be considered part of routine care. Annual assessment of HOMA-IR, fasting glucose and lipids, alongside routine monitoring, can be considered to inform targeted advice and to identify those children and young people who may be at increased risk.

Salivary cortisone , is a better marker of plasma cortisol concentrations than salivary cortisol, which is supported by findings in GRACE 1 (see section 4.3.5) and in previous literature [125]. Normative data are available; however, larger data sets are required to give robust reference ranges in children. Hydrocortisone doses used within this population resulted in total cortisone exposure similar to healthy children and young people. However, non-physiological peaks and troughs are apparent.

5.4.8. Recommendations for future research

Similar to GRACE 1, these glucose data are referenced against normative datasets obtained elsewhere, as are many of the cardiovascular data. A study, with matched controls from the local community may be more informative. Salivary data are compared with healthy controls from the local population.

Newer hydrocortisone formulations including Efmody® aim to replicate the physiological diurnal rhythm of cortisol in people with AI. This medication is currently licensed for use in people with CAH aged 12 years and up. It would be of interest to repeat the study protocol described in this thesis in patients with secondary AI,

treated with MR-HC to assess whether normalisation of the diurnal profile helps to reduce the cardiovascular risk factors described, therefore leading to reduced morbidity and mortality and adulthood.

6. Chapter 6: Retrospective review of local practice in testing the adrenal axis with the low dose short Synacthen test

6.1. Introduction

My clinical research fellow post began in August 2020. This was a challenging time during the COVID-19 pandemic. There was uncertainty regarding recruitment, regarding whether research studies would continue if they were not COVID-19 related and restrictions were in place reducing movement between cities and countries that would be necessary for families to travel for the study visits. It was for this reason other research opportunities were considered. This chapter focusses on data collected relating to LDSSTs performed locally, investigating baseline cortisol concentrations, peak cortisol concentrations and incremental changes based on age, sex and reasons for performing the test. Methods and results and discussion are presented within in this chapter.

Data collection was performed by myself and a medical student, now Dr Selvarajah. Biochemical colleagues Darren Powell and Catherine Collingwood were involved in interpretation and analysis of samples. Descriptive and basic statistics were performed by myself. However, Andrew Titman and Gillian Lancaster, both statisticians helped to perform further statistical testing. Professor Blair, Dr Das, Dr Dharmaraj, Dr Didi, Dr Senniappan were involved in clinical analysis of results and long-term management of the patients involved. Professor Blair oversaw the project. All authors reviewed the manuscript and provided input for the final draft. The published article is referenced here [1] can be found in the appendix 9.12.

The diagnosis of adrenal insufficiency (AI) in childhood is challenging. Details of this can be seen in section 1.3.3.

The LDSST protocol used in our centre since 2008 uses a dose of Synacthen calculated according to body surface area (500ng/m^2body surface area) [193]. Metanalyses consistently report that the LDSST is more sensitive, but less specific than the SDSST [43-46]. In our practice we elected to use the more sensitive test.

Results of those children tested using the simplified LDSST were assessed regularly. There is a risk of over diagnosis and treatment of AI, given the concerns regarding the

specificity of the LDSST. For this reason, the number and characteristics of children diagnosed with AI requiring treatment with daily hydrocortisone were examined. The relationship between age and gender, and baseline and stimulated cortisol concentrations were investigated as it has been suggested that age and gender specific reference ranges should be developed. It has also been reported that an early morning measurement of cortisol can be used to screen children for AI [45, 194, 195], and therefore the relationship between basal and stimulated cortisol was also investigated.

To gain further understanding of the test, children were grouped according to clinical features that may indicate a higher or lower likelihood of a diagnosis of AI. For example, we anticipated that children with isolated fatigue in the absence of known risk factors for AI would be least likely to have an abnormal result, while those treated with extended course of pharmacological doses of glucocorticoids were more likely to have AI.

The aims of this study were (1) to understand how the LDSST was used in routine clinical practice; (2) how often, according to the departmental protocol [193] children were prescribed hydrocortisone either daily or during periods of stress only, and (3) to examine for relationships between cortisol measurements and indication for testing, age and sex.

6.2. Materials and Methods

The protocol was reviewed by and registered with the Alder Hey Children's NHS Foundation Trust audit committee (reference number 6134).

6.2.1. Population

The results of LDSSTs performed in children attending the day care unit or during an inpatient admission in our tertiary children's hospital between [2008 to 2014 (72 months) and 2016 to 2020 (42 months)] were reviewed. Each patient was included only once in the data set. If a child had more than one LDSST only the first test was included.

The following data were collected retrospectively: Age, sex, indication for the LDSST and cortisol concentrations at each time point.

Children were classified into the following groups: respiratory disease requiring treatment with inhaled corticosteroids (ICS), completion of an extended course of pharmacological doses of glucocorticoids for other conditions (iatrogenic AI) including gastrointestinal, malignant, rheumatological and renal disease; structural brain lesions involving the hypothalamus and/or pituitary diagnosed prior to the LDSST; poor cortisol response (peak cortisol <450nmol/L) to a growth hormone stimulation test performed for the assessment of short stature (insulin tolerance tests and glucagon stimulation tests); infants less than one year of age with clinical features of AI; fatigue in the absence of known risk factors for hypothalamic, pituitary or adrenal pathology and children tested because they had one or more autoimmune endocrine pathologies known to be associated with AI, in whom clinical assessment by the lead consultant endocrinologist deemed testing to be appropriate and 'miscellaneous' reasons [193]. Infants were tested when a random cortisol was low during critical illness, prolonged jaundice, recurrent hypoglycaemia and following high dose glucocorticoid use in intensive care.

Details of other pituitary hormone deficiencies were recorded for children with structural brain lesions, diagnosed with AI on the LDSST.

Children with an abnormal cortisol response to a growth hormone stimulation test were subclassified as those with growth hormone deficiency (GHD) and those with a normal GH response. Children treated with inhaled corticosteroids were tested if they were prescribed a dose of >800 micrograms beclomethasone equivalent / day, or children on >500 micrograms of fluticasone for a period of greater than six months, had received frequent 'rescue doses' of oral prednisolone, or had symptoms of AI including poor growth, significant gain in BMI or excessive fatigue. The first time period included some children recruited to one of our previous studies of adrenal function in children with asthma [196], which also recruited children on lower doses of ICS and those without symptoms of AI.

Data were analysed as a single group, and in diagnostic groups that included more than 50 children.

6.2.2. LDSST procedure

Details of the LDSST procedure are given within DataCat: The research Data Catalogue at the University of Liverpool [193]. In brief, on the day of the investigation, an indwelling venous catheter was sited following the application of local anaesthetic cream. A blood sample was collected (time 0). Body surface area was calculated from weight, and the dose of Synacthen was determined from a standard table [193]. One hundred and twenty-five micrograms (0.5ml) Synacthen (Alliance, Chippenham, UK) 0.25mg/ml solution was added to 500ml 0.9%Nacl (final concentration of 250nanograms/ml) and agitated. Five hundred nanograms/1.73m^2 body surface area was administered as a bolus injection directly into the cannula and samples were collected 15, 25 and 35 minutes following Synacthen administration.

6.2.3. Assays

Serum cortisol was measured in the clinical laboratory at Alder Hey Children's Hospital by immunoassay using the Siemens Immulite 2000XPi immunoassay system (Siemens Diagnostics, Camberley, Surrey), an automated immunoassay analyser using reagents supplied by the manufacturer. This assay has an intra- and inter-assay coefficient of variation of <5% and <7% respectively. The same assay was used throughout this time period.

6.2.4. Interpretation of cortisol responses to the LDSST

Children were classified into one of three groups on the basis of the baseline and peak cortisol measurement: (1) baseline cortisol was ≥100nmol/L and peak cortisol ≥450nmol/L were considered to be at very low risk of AI and were classified as 'normal' (2) peak cortisol concentrations in the range of 350 - <450nmol/L were considered to be 'suboptimal' and (3) those in whom peak cortisol was <350nmol/L, who we considered to be a greatest risk of adrenal crisis and were described as 'abnormal'. These values were ascertained from a review of case reports and case series reporting adrenal crisis during ICS therapy in childhood [197-202] . Other than one patient, all other children, who were tested in the non-acute setting had a peak cortisol <350nmol/L and generally below 200nmol/L. For this reason, a cut off value of peak cortisol <350nmol/L was identified to determine those at greatest risk of adrenal crisis. Previous literature reports that a peak cortisol of 500nmol/L in short, healthy children has a false positive rate of 21% for the diagnosis of AI whilst a specificity of 94% has

been reported, using a peak of 415nmol/L [203]. These data informed our definition of a 'normal' peak cortisol being ≥450nmol/L.

Children with a suboptimal response were treated with hydrocortisone (20mg/m^2/day) on 'sick days' only, defined as days that the child was unwell enough to be kept off nursery or school, injuries severe enough to need hospital treatment and surgical procedures. Those with an abnormal result were treated with maintenance hydrocortisone (8-10mg/m^2/day) and sick day doses. If the diagnosis remained unknown after failing the LDSST further investigations were undertaken to assess the cause.

6.2.5. Statistics

Data were recorded in Microsoft Excel and analysed using 'R' version 4.0.2 [204, 205]. A value of 50 was imputed for children whose baseline cortisol was below the limit of detection (50 nmol/L). The relationship between baseline cortisol and peak cortisol showed different gradients for lower versus higher baseline cortisol concentrations. For this reason, quantile regression techniques [206] were used to estimate the 5^{th} and 95^{th} percentile levels of peak cortisol for different values of baseline cortisol. Relationships were assessed using linear regression and adjusted for explanatory variables as required.

6.3. Results

6.3.1. Characteristics

Data from 494 children were analysed. The mean age was 9.5 ± 5.2 years and 295 (61%) were male. All samples across both time periods were amalgamated as there were no changes in the study protocol or in the assay during this time period. Patient characteristics are summarised in Table 6.1.

Table 6.1: Characteristics of paediatric participants undergoing the LDSST by diagnostic category. Data are shown as mean (± SD)

Indication for test (N)	Percentage of all tests in period	Age (years)	Male, n (%)
Whole group (481)	100	9.5 ± 5.2	295 (61)
Inhaled steroids (ICS) (106)	22	10.5 ± 3.6	66 (62)
Pharmacological doses of steroids, not ICS (40)	8	9.7 ± 5.8	20(50)
Structural brain abnormality (136)	28	9.4 ± 1.0	77 (57)
Poor cortisol response to GH stimulation test (29)	6	8.2 ± 4.5	19 (66)
GHD (27)	6	10.6 ± 3.5	21 (78)
Infants (35)	7	0.3 ± 0.3	24 (69)
Fatigue (37)	8	11.7 ± 4.5	19 (51)
Associated autoimmune conditions (13)	3	15.1 ± 1.9	6 (46)
Miscellaneous (58)	12	9.5 ± 5.4	43 (74)

N: number, %: percentage, ICS: inhaled corticosteroids, GH: growth hormone, GHD: growth hormone deficiency.

6.3.2. Cortisol Responses to the LDSST

For the group analysed as a whole, mean (± SD) baseline cortisol was 221 ± 120 nmol/L, peak cortisol 510 ± 166nmol/L and cortisol increment was 210 ± 116 nmol/L. Three hundred and thirty-six (70%) children were classified as having a normal response, 78 (16%) a suboptimal response and 67 (14%) an abnormal response. Table 6.2 summarises cortisol responses to the LDSST as a whole and by sub-group.

Table 6.2: Baseline and stimulated cortisol responses to the LDSST

Indication for test (N)	Baseline cortisol (nmol/L	Peak cortisol (nmol/L)	Cortisol Increment (nmol/L)	Normal (%)	Suboptimal (%)	Abnormal (%)
All CYP (481)	221 ± 120	510 ± 166	210 ± 116	336 (70)	78 (16)	67 (14)
Inhaled steroids (106)	192 ± 94	431 ± 123	241 ± 120	59 (56)	26 (24)	21 (20)
Iatrogenic not ICS (40)	197 ± 91	410 ± 150	216 ± 145	20 (50)	8 (20)	12 (30)
Structural brain abnormality (136)	241 ± 125	553 ± 125	229 ± 121	108 (79)	15 (11)	13 (10)
Poor cortisol response to GH stimulation test (29)	236 ± 86	649 ± 88	391 ± 104	26 (90)	3 (10)	0(0)
GH deficiency (27)	241 ± 85	639 ± 137	382 ± 154	22 (81)	4 (15)	1 (4)
Infants (35)	183 ± 188	465 ± 249	282 ± 224	13 (37)	9 (26)	13 (37)
Fatigue (37)	236 ± 117	583 ± 85	347 ± 134	36 (97)	1 (3)	0 (0)
Autoimmune (13)	305 ± 185	609 ± 185	307 ± 137	10 (77)	3 (23)	0 (0)
Miscellaneous (58)	214 ± 109	525 ± 170	317 ± 162	42 (72)	9 (16)	7 (12)

N: number, nmol/L: nanomoles per litre, %: percentage, CYP: children and young people, ICS: inhaled corticosteroids, GH: growth hormone, GHD: growth hormone deficiency.

6.3.3. Age and gender by diagnostic groups

Figure 6-1 gives a boxplot of the age distributions of each of the diagnostic groups (excluding infants). Age differed significantly between groups ($p<0.001$, based on Kruskal-Wallis test), with children with autoimmune conditions and those with isolated fatigue being older.

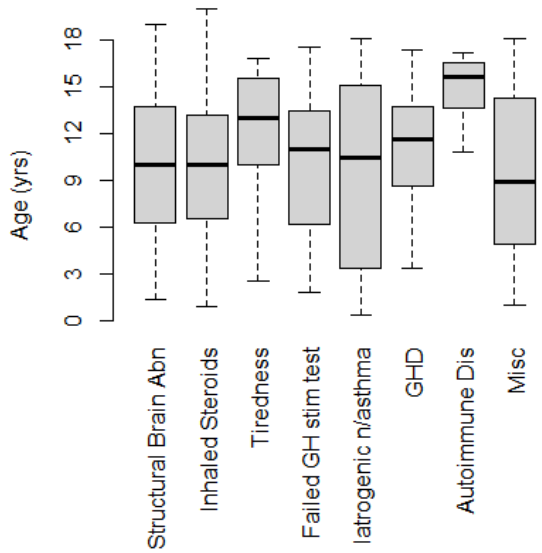


Figure 6-1: Boxplot of age distribution by diagnostic groups

There is also evidence of heterogeneity with respect to gender across diagnosis groups (Pearson Chi-squared test, p=0.015, Table 6.1). There are higher proportions of male children with GHD and in the miscellaneous category and higher proportions of female children in the structural brain abnormality and autoimmune disease groups.

6.3.4. Relationship between baseline and peak cortisol

The relationship between baseline cortisol and peak cortisol is illustrated in Figure 6-2 showing quantile regression estimates of the 5th percentile (q_5) and 95% percentile (q_{95}) of peak cortisol as a function of baseline cortisol.

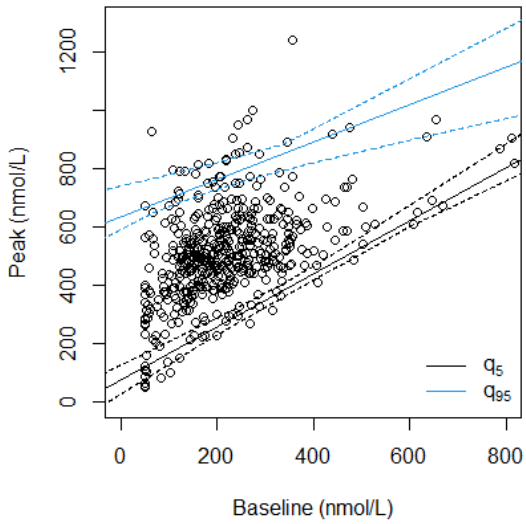


Figure 6-2: Quantile regression estimates of the 5th percentile (q5) and 95% percentile (q95) of peak cortisol as a function of baseline cortisol. Dashed lines indicate pointwise 95% confidence intervals

The 5th and 95th percentile levels of peak cortisol for different values of baseline cortisol are detailed in Table 6.3.

Table 6.3: Estimated model parameters for the quantile regression models of for percentiles of peak cortisol as a function of baseline cortisol

Model for 5th percentile	**Estimate**	**95% CI**
Intercept (α_1)	74.01	47.18, 98.41
Slope (β_1)	0.909	0.866, 0.959
Model for 95th percentile	**Estimate**	**95% CI**
Intercept (α_2)	634.6	597.7, 725.3
Slope (β_2)	0.641	0.460, 0.895

CI: confidence interval.

These data can be used to predict peak cortisol based on baseline cortisol. For example, if baseline cortisol was 200nmol/L the 5th percentile would be 255.8 [(200 x 0.909) + 74] and 95th percentile would be 762.8nmol/L [(200 x 0.641) + 634]. If baseline is 200nmol/L there is a 90% probability that the peak cortisol concentration will be between 255.8 and 762.8nmol/L. The analysis can also be used to determine the estimated level of baseline cortisol for which the 5th percentile of peak cortisol is equal

to a given value. For instance, it is estimated at a baseline cortisol level of 418 nmol/L (95% CI: 384, 435), there is a 5% chance of a peak cortisol level below 450nmol/L. The inclusion of quadratic terms in the quantile regression was investigated but did not result in a significant improvement of fit.

All children with a baseline cortisol of ≥419 nmol/L had a peak cortisol value of ≥450 nmol/L. In contrast, one patient whose baseline cortisol value was below the limit of detection (<50 nmol/L) had a peak cortisol value of 673 nmol/L. As such, there is no baseline cortisol value below which all children in the dataset had a peak below 350 nmol/L, the value at which the children would be classed as failing the LDSST.

6.3.5. Baseline cortisol

6.3.5.1. Relationship of baseline cortisol levels with age and gender

Age was related to baseline cortisol. Baseline cortisol increased by 2.7% (95% CI: 1.8%,3.7%) per one-year increase in age. This relationship persisted after diagnostic group and gender were included as explanatory variables within the linear regression, although the magnitude of the effect decreased to 1.9% per year (p<0.001, 95% CI: 0.8%, 3.0%).

Baseline cortisol measurements were 11.5% higher in girls than in boys (p=0.030, 95% CI: 1.1%, 23.1%).

6.3.5.2. Differences between diagnostic groups

Differences in mean baseline cortisol values were observed between diagnostic groups, even after adjusting for differences in age and gender (F-test p=0.006). GHD and children with an autoimmune condition associated with AI, had the highest baseline cortisol values, with infants and those with structural brain abnormalities having the lowest values.

6.3.6. Peak cortisol

6.3.6.1. Relationship of peak cortisol levels with age and gender

There was no effect of age on peak cortisol (either with or without controlling for diagnostic group). However, there was a significant difference with gender, with girls having peak cortisol levels 60 nmol/L (95% CI: 31.4, 88.6, p<0.001) higher than boys after adjusting for diagnostic group and age. This effect also persisted after including baseline cortisol as a predictor for peak cortisol (effect of gender 45.7nmol/L (95% CI:

20.15, 71.22, p<0.001) if age, diagnostic category and baseline cortisol level was included).

6.3.6.2. Differences between diagnostic groups

Peak cortisol values between diagnostic groups differed, even after adjusting for differences in age, gender and including baseline cortisol as a predictor (F-test p<0.0001). The groups with the lowest peaks are those treated with pharmacological doses of glucocorticoids, with structural brain abnormalities and infants, while the highest peaks occurred in children with isolated fatigue, a diagnosis of GHD in the absence of symptoms of AI and those with autoimmune disease. All children who failed the LDSST who had structural brain lesion, had at least one additional pituitary hormone deficiency [193].

6.3.7. Increment in cortisol concentration

6.3.7.1. Relationship of incremental increase in cortisol concentration levels with age and gender

Age was associated with a decrease in the increment in cortisol concentration between baseline and peak (5.8 nmol/L reduction per year of age (95% CI: 2.9, 8.6, p<0.0001). As anticipated, given the relationships found in Sections 2.1 and 2.2. The increment in cortisol was 28.9 nmol/L (95% CI: 3.2, 54.7, p=0.03) higher in girls than boys after adjusting for age and diagnostic category.

6.3.7.2. Differences between diagnostic groups

Mean increment in cortisol also differed between diagnostic groups (F-test p<0.0001), with those treated with pharmacological doses of glucocorticoids, and structural brain abnormalities having the smallest increments and those with isolated fatigue and those with autoimmune disease having the largest increments.

6.4. Discussion

We believe this is the largest dataset of cortisol profiles in children undergoing the LDSST reported to date. Analysis of such a large cohort has enabled relationships between baseline and peak cortisol concentrations and indication for testing, age and sex to be explored. We see trends reported in previous work, including higher concentrations of cortisol in girls and older children [146, 207].

In this cohort of children, baseline cortisol concentrations could not be used to identify children who pass or fail the LDSST in a clinically helpful manner. It is likely that the baseline sample, collected as close to 09.00 as possible, did not capture the peak of the cortisol awakening response in most children, and we did not document the time of waking on the day of the test. Collecting a blood or saliva sample at home, half an hour after waking, may identify the true peak cortisol of the awakening response and be more informative.

Baseline cortisol was related to age and gender, with older children having modestly higher baseline cortisol concentrations than younger children, with an incremental increase with each one-year increase in age. These observations are consistent with previous studies [207]. Peak cortisol was not affected by age, and correspondingly, incremental change in cortisol levels from baseline to peak decreased as age increased. Although these differences reach statistical significance and may be of interest in understanding the physiology of the maturing HPA axis, we suggest they are too small to justify the complexity of generating and using age related reference ranges. These data are consistent with a previous study in a large cohort of children with asthma, treated with ICS [208].

Baseline and peak cortisol measurements and incremental increase in cortisol was higher in girls than boys. The difference in peak cortisol between girls and boys is similar to the sex difference we reported in an asthma cohort, tested using the same LDSST protocol, 51.9nmol/L higher in girls than boys, 95%CI=-84.81, -18.89, p= 0.002 [196]. This difference was not explained by age, and therefore pubertal status, or higher baseline cortisol concentrations.

Baseline plasma cortisol differed by sex. However, in chapter 3, no difference in early morning salivary cortisol concentrations can be seen. Plasma cortisol measures both free cortisol and cortisol bound to proteins. Cortisol binding globulin or other binding proteins may differ between sexes. These studies also compared different populations. SMILE (chapter 3) assessed salivary cortisol in healthy children whilst the LDSSTs were performed on those suspected to have AI. These baseline measurements

include both those who passed and those who failed the test. It would be interesting to assess only those who passed and are therefore thought to have no disease of the HPA axis and compare them with the salivary results of healthy children.

We observed that those we anticipate being at greatest risk of AI (known structural brain abnormalities and following pharmacological doses of glucocorticoids) had the lowest cortisol concentrations at baseline and following Synacthen stimulation even after adjusting for differences in age and gender, and when including baseline cortisol as a predictor for peak cortisol. Our data do not allow us to comment on the sensitivity and specificity of the test in the absence of reference data from a healthy population, but it is reassuring that our observations make clinical sense.

It was anticipated that children treated with pharmacological doses of glucocorticoids for prolonged periods of time are likely to have had adrenal suppression. These children had been treated by specialists from other disciplines in our hospital, following their own weaning regimens prior to testing. Some patients underwent multiple tests during adrenal recovery. Only the first test was included in our study and the period between the completion of glucocorticoid therapy and the LDSST is likely to differ significantly between children. High rates of adrenal suppression have been described in children following prolonged glucocorticoid therapy [209-211], and recovery of the HPA axis in children treated with ICS using this test protocol has also been described [208].

After children with iatrogenic AI, children with structural brain lesions were most likely to have AI. Each child who was diagnosed with AI via this test, had evidence of another pituitary hormone deficiency, which is consistent with the sequence of pituitary hormone loss, in which ACTH deficiency is generally preceded by growth hormone deficiency. In a previous cohort of children with brain tumours, 10.3% had evidence of ACTH deficiency [212]. This is similar to the number of children treated with daily hydrocortisone therapy on the basis of the LDSST result in our cohort, which also included children with congenital defects of the pituitary, or following traumatic brain injury who may be at greater risk of ACTH deficiency [213].

Children with an impaired cortisol response to a GH stimulation test were evaluated further with the LDSST. Most children in this category had a normal GH response and had undergone a glucagon stimulation test. If the child had undergone an insulin tolerance test, the LDSST was performed only if the child had no symptoms of cortisol deficiency. In this cohort of children, 20 (87%) children had a normal LDSST result, suggesting that the glucagon stimulation test may be less specific for the diagnosis of AI, and we have since revised the threshold at which the peak cortisol is considered to be abnormal on the glucagon stimulation test from >550 nmol/L to >350 nmol/L [214].

In a previous study, using basal serum cortisol <198.7nmol/L with no significant increase during the insulin tolerance test, a much higher prevalence of ACTH deficiency was reported in children with GHD and a normal pituitary on MRI [214]. This is likely to reflect differences in the patient cohort and diagnostic tests.

The difficulties of diagnosing AI in infants, in whom a number of perinatal factors are likely to influence activity of the HPA axis, is well documented. In this cohort, we found baseline and peak cortisol concentrations were lower in infants than in all other diagnostic categories, other than those with structural brain abnormalities. In a previous study, 43% of infants demonstrated an abnormal response to a standard dose Synacthen test [215], very similar to the number of infants treated with daily hydrocortisone in our cohort of children. In this previous study, 69% of infants with an abnormal result had no identified pathology, and 90% of these children had a normal response to the standard dose test at a later date [215], emphasising the importance of repeat testing in infants.

Peak cortisol was highest in those children with fatigue, but no other clinical symptoms or signs of AI, no known pituitary or hypothalamic lesion, and no known risk factors for AI. The majority of children in this group had a normal response to LDSST. These data give some insight to the likely specificity of the LDSST and suggest testing for AI is unlikely to be informative in this group of children. We observed similar results in the group of children who were tested routinely, because they had one other autoimmune condition known to be associated with AI, but were not necessarily symptomatic. These data suggest that testing should only be performed in children with additional features of AI.

It is a weakness of this study that there are no reference data from healthy children. It is difficult to undertake invasive studies in healthy child volunteers, and our definitions of a 'normal, 'abnormal' and 'suboptimal' response have been derived from historical data from cohorts of children with asthma [208, 216]. Therefore, it is not possible to state with confidence whether we have accurately, under or over diagnosed AI. However, the relatively small number of children treated with daily hydrocortisone on the basis of the LDSST, and the clustering of children with an abnormal result in diagnostic groups we consider to be at greatest risk of AI, suggest that overdiagnosis and treatment is unlikely to be very common.

All LDSSTs were performed by endocrine nurse specialists in a single medical day care unit, or on inpatient wards, according to a protocol [193] which is carefully designed to address concerns regarding the reliability of dosing and the risk of absorption to plastics. It is likely that results would be less reproducible in centres where the test is performed less commonly, or by less specialised staff.

The development of the salivary Synacthen test is an extremely positive development, as the use of non-invasive testing is likely to enable the development of reference ranges in the paediatric population.

6.5. Conclusion

In conclusion, data from this large cohort of children shows the results of the LDSST are consistent with previous studies, that the risk of over diagnosis and treatment of AI in this clinical setting is probably low, and that clinical acumen is important in the selection of children that do and do not require testing. The number of children treated with daily hydrocortisone was modest, and in most diagnostic groups, not dissimilar from data reported previously.

We suggest the uncertainty rests in the group of children with a 'suboptimal' test result, in whom we recommend hydrocortisone during periods of stress only. This conservative approach was adopted with the introduction of the LDSST protocol, to avoid unnecessary daily treatment, but to protect against adrenal crises in children with a peak cortisol response between the new threshold (350nmol/L) and historical thresholds (450-500nmol/L). We do not have strong evidence for this recommendation, and further work needs to be done to refine these thresholds.

7. Chapter 7: Summary and further work

7.1. Findings

This thesis explores and develops themes reported previously: (1) that patients with AI are at increased risk of cardiovascular disease and (2) that cortisol profiles in patients treated with hydrocortisone are non-physiological. This work considers how these observations may be linked, by describing salivary cortisol and cortisone profiles, and examining for associations between these profiles and markers of cardiovascular and metabolic health. Novel data, describing additional adrenal biomarkers measured in the saliva of healthy volunteers and those with primary AI are also reported. The background for these studies is outlined in Chapter 1. Altered glucose metabolism has been described previously in smaller studies, over several days, using blood glucose and short-term use of CGMS [65, 66, 68]. It has been reported on many occasions that AI is associated with cardiovascular risk factors including raised BMI, raised BP, increased CIMT measurements and evidence of metabolic syndrome [85].

The second chapter of this thesis reports an audit of the care of children and young people with CAH at Alder Hey Children's Hospital and compares this to international guidance [94]. Participants for the GRACE 1 study were recruited from this clinic population. Salivary cortisol and cortisone data from a small pilot study have been published previously [146]. A larger dataset is described in chapter three. Alongside this, novel data are presented of salivary adrenal biomarker concentrations including testosterone, A4, 11KT and 11OHA4 from a cohort of healthy children aged 5 – 18 years of age. Chapter four and five report the largest studies to date of glucose regulation over a seven-day period in children with primary and secondary AI, respectively. Secondary outcomes highlight the increase in cardiovascular risk factors seen in paediatric patients with AI. Salivary biomarkers were measured in the participants with primary AI, which were then compared to those found in healthy children. Finally, the work for this thesis was undertaken during the COVID-19 pandemic, when recruitment to non-COVID related studies was uncertain. Therefore, chapter six shows data analysed when recruitment to the GRACE 1 and 2 studies was uncertain, from 494 LDSSTs performed locally investigating baseline, peak and

incremental cortisol concentrations by age, gender and indication for testing. These data have been published recently [1].

7.2. What have we learnt from these studies?

7.2.1.1. Glucose metabolism

At the outset of this project, it was anticipated that children with AI, treated with hydrocortisone, would be at increased risk of hypoglycaemia. It was thought that this was particularly likely to occur in the youngest children, who take the last dose of hydrocortisone relatively early in the evening, and fast for longest overnight. Although one child with primary AI showed evidence of hypoglycaemia (<3mmol/L for more than 2% of the time), this improved with a modest increase in hydrocortisone dose (9.2 to 9.8mg/m^2/day). This child was four years of age and had a history of an adrenal crisis without an obvious precipitant. No other child had evidence of hypoglycaemia. In fact, rather than hypoglycaemia, hyperglycaemia was evident. This was particularly evident after a dose of hydrocortisone. Unfortunately, we did not collect information regarding meal times, and it is possible that a meal was ingested concurrently. GRACE 2 was powered to determine whether hyperglycaemia occurs more frequently in children and young people with secondary AI than the reference population. Although no participants in either study had evidence of hyperglycaemia (>10mmol/L for more than 2% of the time), there was a clear trend for glucose concentrations to be higher in both study cohorts than in healthy children. The clinical significance of this is unknown. These data using CGMS over a seven-day period suggest that when children with AI are well and are treated with adequate hydrocortisone replacement, hypoglycaemia does not seem to be a concern. However, further assessment of the effects of higher mean glucose concentrations are warranted.

7.2.1.2. Cardiovascular outcomes

A further focus of this thesis was to learn more about the cardiovascular risk profile of children and young people with AI, as it is possible that early and targeted intervention during the childhood and adolescent years may improve long term health and life expectancy. It is therefore interesting to note that higher glucose concentrations, even within the normal range, can be associated with increased risk of cardiovascular disease, stroke and all-cause mortality [167]. It is unknown as to whether the small increase in mean glucose concentrations reported in these studies has a clinically

significant effect on long term health. However, it can be hypothesised that 0.5mmol/L higher for each year of life may have detrimental effects. This observation requires further research. Adults with primary and secondary AI are at increased risk of diabetes and insulin resistance [192]. These data may shed some light on the early evolution of this co-morbidity.

Cardiovascular events in children are rare, therefore markers of subclinical cardiovascular disease are required. Hypertension was seen in primary AI, particularly in younger children on high doses of fludrocortisone for their body weight. Insulin resistance is prevalent in those with both primary and secondary AI, with severe insulin resistance in the secondary AI group. Abnormal lipid profiles can be seen in both groups. VWF antigen and activity have been shown to be associated with increased cardiovascular risk in the adult population[83, 84]. Some children show higher levels antigen and activity of VWF. FMD is also reduced in a high proportion of children within both populations. Most cardiovascular risk factors are seen clustered within the same participants while others have no risk factors. Most children with clustering of risk factors have a higher BMI SDS. Whether obesity is the first event in the evolution of cardiovascular disease, or occurs concurrently as one other manifestation of non-physiological cortisol exposure is unknown. However, some healthy weight participants show evidence of increased cardiovascular risk.

7.2.1.3. Salivary biomarkers

7.2.1.3.1. Salivary cortisol and cortisone

In contrast to data from healthy children, it is likely that salivary cortisol is not a reliable indicator of serum cortisol concentrations in patients with AI due to high rates of contamination by hydrocortisone. Salivary cortisone is more informative, and displayed a diurnal pattern when measured two-hourly throughout the day in the GRACE 1 study, regardless of the timing of hydrocortisone doses. In an individual level, salivary cortisone measurements did show peaks and troughs. However, once all data were amalgamated these peaks and troughs were lost. It is reassuring that total cortisone exposure is similar to that of healthy volunteers at the doses used to treat AI. When measurement of salivary cortisone was timed with a dose of hydrocortisone in GRACE 2, those peaks and troughs persist when data from the whole group are

analysed together. The total cortisone exposure throughout the day is similar between the cohorts.

7.2.1.3.2. Salivary testosterone, A4, 11KT and 11OHA4

Salivary testosterone and A4 are part of the classical steroidogenesis pathway that has been used for monitoring of adrenal disease historically. 11oxC19 steroids have recently been described, but little are known about them. Within this thesis, data from healthy children aged 5 – 18 years have been described. More participants will be recruited to this cohort with an aim to generate robust reference ranges. These will form the foundation for further research into the utility of these biomarkers for the diagnosis and monitoring of treatment in adrenal disease. Salivary 11KT concentrations were lower in children with Addison's disease but higher in children with CAH. 11OHA4 concentrations, another 11oxC19 steroid are also higher in those children with CAH. Further research should assess whether salivary 11KT concentrations are associated with disease control in CAH, and may be an alternative method of monitoring treatment efficacy to the more commonly used 17OHP blood spot profiling. Target ranges, and how these relate to age and sex, will need to be established.

7.2.1.4. LDSST in the diagnosis of AI

Finally, this thesis considers the outcomes to the LDSST in children and adolescents by the indication for testing. There are controversies surrounding the best way to test the HPA axis in children. Insulin tolerance tests are thought to be the 'gold standard' test. However, they are unpleasant and not without risk, some fatalities having been reported [217]. Testing the HPA axis by measuring adrenal responsiveness to the synthetic copy of ACTH (Synacthen) is used commonly in paediatrics. However, there is considerable controversy about the optimal testing protocol. Standard Synacthen tests use doses of Synacthen that result in supraphysiological stimulation of the adrenal gland whilst low dose Synacthen tests are thought to represent a more physiological dose. LDSSTs are considered to be more sensitive but less specific than the standard dose test [43-46], leading to the possibility that some children will have false positive results and are therefore treated with hydrocortisone unnecessarily.

This retrospective review of LDSSTs showed no baseline cortisol concentration could be used to define whether a child passed or failed the LDSST. Baseline cortisol was related to age and higher in girls than boys. There were strong associations between the likelihood of having an abnormal result and the indication for testing, which made clinical sense. Furthermore, the number of children who were treated with daily hydrocortisone on the grounds of the LDSST results was relatively modest, suggesting that overtreatment is probably not common. The development of non-invasive Synacthen tests, using intra-nasal Synacthen and salivary sampling may enable reference ranges to be developed from healthy child volunteers [218]. On reopening the Salivary markers in healthy children (SMILE) study, we hope to provide robust reference ranges to help guide diagnosis and management of AI in the future.

7.3. Clinical implications for care

Studies described within this thesis confirm previous reported data that cardiovascular risk factors are evident in both populations of children and young people with AI. Healthy lifestyle advice, weight and blood pressure management, guidance on avoiding smoking and excess alcohol consumption must be discussed regularly with patients with primary AI to reduce effects of modifiable risk factors and improve morbidity and mortality later in adulthood.

The data reported in this thesis indicate that a range of adrenal biomarkers can be measured in saliva and show a diurnal profile. While further work is required to define the reference ranges for all, and clinical utility of the more novel biomarkers, we can state with confidence that salivary cortisone is a more useful measure than salivary cortisol in the clinical setting. In non-treated children, salivary cortisone is detectable at all timepoints, while cortisol is often undetectable in afternoon and evening samples and in treated patients, hydrocortisone contamination results in falsely elevated measurements.

Until we have greater understanding of the clinical significance of the modest increase in glucose measurements observed on CGM, and an effective intervention, routine monitoring cannot be recommended. It was reassuring that only one child had significant hypoglycaemia, who had reported symptoms previously.

7.4. Future research

7.4.1. Future research into glucose metabolism in AI

Future research to confirm the altered glucose metabolism seen in these studies should be considered in a larger and different clinic population. CGMS data from the first 24 hours were excluded. It is understood that CGMS data are more accurate after the first 24 hours therefore any interventions that are also being analysed should be at the time when the glucose data are most accurate. ABPM, hydrocortisone medication, a food diary and salivary sampling could occur on day three to ensure that all data can be assessed together. For example, salivary hormone measurements, periods of hypotension, hypertension, hypoglycaemia or hyperglycaemia can be described within the same 24 hours. Direct effects may be determined this way.

7.4.2. Future research in pharmacogenomics in AI

Pharmacogenomic studies associated with responses to glucocorticoids have been described. This can be seen in those treated with glucocorticoids for hypocortisolaemia and also when used to treat other systemic disease. Some children respond to glucocorticoid treatment to treat diseases such as nephrotic syndrome, whilst some do not. Genetic polymorphisms may explain why some children have altered glucose metabolism, higher propensity to obesity, or increased risk of cardiovascular disease. Pharmacogenomic studies may show that those with clustering of cardiovascular risk factors are either more sensitive to glucocorticoids, or have other gene changes that increase their risk of cardiovascular disease.

7.4.3. Future research in newer MR-HF

Newer, modified release hydrocortisone formulations have shown smoother diurnal serum cortisol profiles, with positive outcomes for Plenadren® in studies undertaken in adults. Studies have shown reductions in systolic BP, diastolic BP, mean body weight and BMI in adults with primary and secondary AI after 12 weeks of treatment [219]. Guarnotta et al have also shown reductions in BMI, waist circumference, HbA1c, increased HDL-c and reduced circulating insulin in those with pre-diabetes shown by impaired fasting glucose [220]. It seems that these newer preparations may reduce the risk of cardiovascular disease. This may be particularly true if treatment is started in childhood, but this is yet to be seen. Research assessing salivary profiles of cortisol, cortisone and other adrenal biomarkers would be of interest to assess whether

salivary cortisone showed a more physiological pattern using this medication. Alongside this, cardiovascular risk factors can be assessed. Longitudinal studies, with matched controls, from childhood into adulthood would be useful to assess lifetime risk. Cardiovascular risk factors may show reversibility including BMI, glucose regulation, BP and improvement of FMD, which provide surrogate markers of improved cardiovascular status.

7.4.4. Future research to assess the utility of salivary cortisol and cortisone in other clinical settings

Salivary biomarkers have potential to be used clinically for both the diagnosis and management of AI. Robust reference ranges are required. SMILE has recently reopened and is currently recruiting for children and young people aged 5 – 18 years of age. Salivary sampling in neonates and children aged less than five years may prove useful as an extension to this. Salivettes designed specifically for younger children were used for those less than 5 years of age in the GRACE 1 study and were tolerated well. Healthy neonates often have low random cortisol concentrations measured in serum which may be associated with lower binding proteins that are seen in neonates and acutely unwell children. Assessment of normative salivary cortisol and cortisone concentrations would help to guide diagnosis of AI in neonates. In scenarios when binding proteins are likely to be low, for example during critical illness, or increased, for example during treatment with oestrogen, salivary measurement of free cortisol and cortisone may provide a better marker of cortisol activity than total cortisol concentrations measured in plasma.

7.4.5. Future research in salivary adrenal biomarkers

Newer salivary adrenal biomarkers, such as the 11oxC19 could be measured comparing the effect that MR-HC has on the salivary profile as a cross over study. Alternatively, this could be compared against those children on SF-HC within the studies presented in this thesis. Longitudinal studies following patients with CAH, whilst assessing salivary 11oxC19 steroids could then be performed. Outcome markers could include height, growth velocity, bone age delay or advancement, symptoms and signs of virilisation, regularity of menses or evidence of TART to assess control of CAH. No one indicator can be used alone to indicate “good control”. Alongside this, cardiovascular markers such as BP, CIMT, FMD, HOMA-IR, leptin, von Willebrand

antigen and activity could be obtained to assess their effect on cardiovascular risk as well as disease control in CAH. Adrenal biomarkers such as 11KT and 11OHA4 may also be useful for diagnosing conditions such as adrenarche and polycystic ovarian syndrome. Further research will be needed to address whether this is plausible.

7.4.6. Future research into interventions to reduce cardiovascular risk in children and young people with AI

Research now needs to progress from reporting the increased risk of cardiovascular disease, to designing interventions to mitigate the risk. Interventions such as weight management advice, dietetic input and psychological support such as those that are becoming more widely available for children with complications of excess weight could be considered. Modifiable cardiovascular outcomes can be used as endpoints. Currently, these services are not generally available for children with AI unless they have significant weight gain. Preventative strategies may be even more useful. We also need to consider the current obesity pandemic, as those children with AI may have similar lifestyles to children with no other health conditions. Intervention for those children with AI may need to occur earlier than guidelines set out for otherwise healthy children. Specific strategies will also be needed for those with concurrent hypothalamic obesity.

8. References

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9. Appendices

9.1. Example of participant information leaflet and consent form (parent version) for GRACE 1

Glucose Regulation and Cardiovascular Health in Adrenal Insufficiency

INFORMATION LEAFLET FOR CHILDREN AGED 9-12 YEARS

Version 5 2nd November 2020

Your Mum or Dad or carer have talked to your doctor a research study.

Why is the research study being done?

We want to learn more about the health of children who take hydrocortisone medicine because their adrenal glands don't work.

The first thing that we want to do is to check your sugar levels. We think that some children might have low sugar levels, especially while they are sleeping. If we find out that your sugar levels are low, we can change your treatment to make them better.

You might remember that when you come to clinic, we measure your blood pressure. We would also like to check your blood pressure levels while you are awake during the day at home or at school and while you are sleeping.

The last thing we would like to do is to check how stretchy your blood vessels (the tubes that carry blood around your body) are.

If your blood pressure was different to other young people, or if your blood vessels weren't as stretchy, it wouldn't cause you any problems now, but we might give you some treatment to make sure it doesn't cause you a problem when you are an adult.

What will happen to me?

You will come to visit us at Alder Hey for a blood test, just as you do every year. This visit will be a little bit different because you cannot eat anything before you come to Alder Hey, but we will give you breakfast after your scans and your blood test. The scans will be of the blood vessels in your arm and your neck. We do need you to sit

still for the scan. We will put a cold jelly on your arm to help with the scan, but these scans won't hurt you

We will show you a small machine that measures your sugar levels. You can see a picture of it just below. It sticks to your tummy or the top of your bottom. It stays there for a week. We will put a blood pressure cuff on your arm for you to wear for the rest of the day and while you are asleep at night. If you are 9 years old we may not need you to wear the blood pressure cuff. We will decide on the day. We will ask you collect samples of your spit throughout that day. We will show you how to do this.

You will be ready to go home or back to school by lunchtime.

Who is doing this research?

It is being run by doctors from Alder Hey Children's Hospital and scientists at the University of Liverpool.

Has anyone checked that this study is ok to do?

Before any research can happen, it must be checked by a group of people called a Research Ethics Committee. They make sure that the research is fair. Your project has been checked.

Who do I ask about this?

Your Mum or Dad or carer will be given lots of information. If they are not sure, the doctor who gave you this information leaflet can tell you more.

9.2. Example of consent form used for GRACE 1

Centre Name and Number:	
Patient's study number:	
Patient's initials: _ _ _	Patient's Date of Birth: _ _/_ _ /_ _ _ _

Glucose Regulation and Cardiovascular Health in Adrenal Insufficiency

CONSENT FORM FOR RESEARCH

For child deemed to have Gillick competence

Version 3.0, 14th September 2020

Name of researcher:	**Please initial box**
1. I confirm that I have read and understand the information sheet for children aged <16 years with Gillick competence (version 4.0), dated 14-9-20 and 'Additional information for child' leaflet, (version 2.0), dated 14-9-2020 for the above study and have had the opportunity to ask questions.	☐
2. I understand that participation is voluntary and that and that I am free to withdraw at any time, without giving a reason, and without my medical care and legal rights being affected.	☐
3. I agree to having additional tests being performed on their annual review bloods	☐
4. I consent to ultrasound imaging being performed.	☐
5. I agree to have a continuous glucose monitor inserted for 7 days, to look after it to the best of my ability and return it after 7 days to make data available.	☐
6. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the research team, regulatory authorities, sponsor, or from the NHS Trust,	☐

where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records for this purpose.

7. I understand that my medical data will be collected for this study and may be used to develop new research and that data protection regulations will be observed. ☐

8. I understand that information about me (including names) will be kept strictly confidential and that no personal information will be used in the study report or other publications. ☐

9. I agree to take part in the above study. ☐

Name of patient _____

| --- | --- | --- |
Name of child	Date	Signature
Name of parent or guardian	Date	Signature
Name of person taking consent	Date	Signature

9.3. Standard operating procedure for carotid intima media thickness

Purpose:

To establish guidelines for the procedure of measuring the intima-media thickness of the carotid artery.

Definitions:

CIMT – Carotid Intima-Media Thickness

USS – Ultrasound

DPP – Debut Professional Programme

Procedure:

Before patient arrives:

1. Collect clean equipment (USS scanner, laptop, BP cuff, stethoscope, gel)
2. Connect Avio monitor to laptop with DPP
3. Press Pre-set and Carotid mode on USS machine
4. Wash hands

Once patient has arrived:

5. Introduce yourself
6. Confirm with patient that they have fasted (including no caffeine)
7. Explain procedure and gain consent
8. Enter anonymised unique patient identifier into USS machine
9. Place gel on USS probe
10. Ask assistant to dim lights
11. Ensure that you are in comfortable position before you start scanning
12. Use red area of USS probe to locate brachial artery – Grey line should be at the top
13. Press Colour doppler on USS machine
14. Set the depth on USS machine
15. Lightly compress vessel with USS probe to confirm presence of artery
16. Set focus on USS machine

17. Turn Colour doppler on USS machine off
18. Both top and bottom intima should be visible on the image (if possible)
19. The carotid blub should be visible
20. Press acquire to take more images (3 images)
21. Provide the participant with tissues to remove any excess gel
22. Thank participant and make them aware that it is the end of the procedure
23. Turn on lights
24. End exam on USS machine to save carotid images

Once patient leaves:

25. Export data before turning off machine
26. Clean and put away used equipment
27. Wash hands

Data analysis:

28. Search patient
29. Click on study
30. Press open study
31. Click on image
32. Open selected image on QLAB
33. Press IMT
34. Select Right - Mid - CCA
35. Find area of interest (approximately 0.5cm from the carotid bulb)
36. Measure a 10mm interval
37. Ensure that success is 95% or above (can reduce to measuring 8mm if needed)
38. Press Acquire to save to study images
39. Press X to get to the next image
40. Repeat steps on 30-28 images 2 more times
41. Calculate an average from these measurements
42. Record on case record form and database

9.4. Standard operating procedure for the assessment of flow mediated dilatation of the brachial artery

Purpose:

To establish guidelines for the procedure of measuring brachial artery flow-mediated dilation.

Definitions:

FMD – Flow Mediated Dilatation

BP – Blood pressure

USS – Ultrasound

DPP – Debut Professional Programme

TGC – Time Gain Compensation

Procedure:

Before patient arrives:

1. Collect clean equipment (USS scanner, laptop, gel)
2. Connect Avio monitor to laptop with DPP
3. Press Pre-set and Arterial mode on USS machine
4. Wash hands

Once patient has arrived:

5. Introduce yourself
6. Confirm with patient that they have fasted (including no caffeine)
7. Explain procedure and gain consent
8. Enter anonymised unique patient identifier into USS machine
9. Measure seated BP in the participants left arm, making a note of the systolic BP reading
10. Ask the patient to rest for 10 minutes
11. Ask the participant to lie in a comfortable position on the bed
12. Ensure that the participants right arm is comfortable and supported
13. Place BP cuff on right forearm, midway between the wrist and elbow

14. Palpate the participants brachial artery
15. Place gel on USS probe
16. Ask assistant to dim lights
17. Ensure that you are in comfortable position before you start scanning
18. Use red area of USS probe to locate brachial artery – Grey line should be at the top
19. Press Colour doppler on USS machine
20. Set the depth on USS machine
21. Lightly compress vessel with USS probe to confirm presence of artery
22. Set the focus on USS machine
23. Remove colour doppler on USS machine
24. Turn on the pulse wave (x2) on USS machine
25. Turn down volume on the USS machine
26. Turn duplex on
27. Move the blue arrow on USS machine so that it is not interfering with the measurable area of the artery
28. Ensure that the arrow is pointing towards the artery (270 degrees) → Angle adjust 60
29. Press spectral invert on USS machine
30. Move baseline on USS machine
31. Change the scale on USS machine
32. Ensure the gain of the image is OK
33. Adjust TGC on USS machine for contrast and clarity of image
34. Check that the participant is comfortable
35. Check you are happy with the image of your artery
36. Press record on DPP
37. Wait 1 minute, holding USS probe on identified artery
38. Inflate BP cuff to 50 mm Hg above the earlier systolic BP reading
39. Leave cuff inflated for 5 minutes, maintain brachial artery image
40. Check on participant regularly and give countdowns (i.e. half way)
41. Deflate BP cuff at the end of the 5-minute period
42. Keep recording the brachial artery for a further 2 minutes
43. Stop recording on DPP (total time recording should be 8 minutes. Leave a 5 second grace period before stopping the recording).

44. Remove USS probe and BP cuff from participant's arm
45. Provide the participant with tissues to remove any excess gel
46. Thank participant and make them aware that it is the end of the FMD procedure
47. Turn on lights
48. Press end exam on USS on machine

Once patient leaves:

49. Export data before turning off machine
50. Clean and put away used equipment
51. Wash hands

9.5. Summary tables for participants of GRACE 1 by diagnosis

Table 9.1: Table summarising patients with salt losing CAH

Study ID	002	004	006	007	008	009	010	012	013	014	015	021	023	024
Baseline Characteristics														
Age (years)	4	5	5	6	4	3	2	12	5	6	7	6	17	16
Sex	M	F	F	F	F	F	M	M	F	M	F	M	M	M
Genotype	21OHD	21OHD	21OHD	21OHD	21OHD	21OHD	21OHD	21OHD	21OHD	21OHD	3β-HSD	21OHD	21OHD	21OHD
Ethnicity	WC	WC	EE	WC	WC	WC	WC	WC	WC	A	Mi	WC	WC	WC
Relevant History														
Antenatal history	GDM	-	Dex	-	-	-	-	-	-	-	-	-	-	-
Smoking history	-	-	-	-	Parental	-	Parental	-	-	Parental	-	-	Parental	Yes
CVD in FDR	-	-	-	-	-	-	-	-	-	Yes	-	-	-	-
FH/participant ↑ lipid	-	-	-	-	-	Dad	-	-	-		-	-	Fhx: FH	FH
Other											Barrter's	-		Epilepsy
Hydrocortisone ($mg/m^2/day$)	9.2	9.2	10.8	11.1	9.9	9.4	9.8	12.5	9.2	11.1	10.2	8.7	9.5	9.1
Fludrocortisone ($mcg/m^2/day$)	211	57.5	269.2	104.4	211.3	234.4	223.2	71.4	153.8	230.3	34.9	174.4	35.7	34.1
Glucose parameters on CGMS														
Mean glucose (mmol/L)	4.9	7.0	-	6.1	5.8	7.0	5.3	6.5	Outlier	5.8	5.8	6.2	5.2	5.6
% glucose<3mmol/L	0.0	2.3	-	0	0	0.0	0.0	0.0	Outlier	0.0	0.1	0.0	0.0	0.0
% glucose>10mmol/L	0.0	0.0	-	0	0	3.2	0.0	0.0		0.0	0	0.9	0.0	0.9
Cardiovascular outcomes														
Insulin resistance				90^{th}-95^{th}				75^{th}-90^{th}		75^{th}-90^{th}	$>97^{th}$	$>97^{th}$	97^{th}	
↑ VWF antigen and activity					Low		TNP			Low	Yes			High
↑ Leptin (≥85^{th} centile)										75^{th}-90^{th}		75^{th}-95^{th}	$>97.5^{th}$	$>97.5^{th}$
BMI SDS	0.6	0.2	-0.1	-0.3	0.7	-0.1	0.5	-0.3	-0.2	-0.4	-0.7	0.7	2.3	3.2
↑ Renin	Low					Low	Low							Low
Clinic HTN (≥95^{th} centile)	Yes	Yes	Yes			Yes	Yes					Yes		Yes
Ambulatory HTN (≥95^{th} centile)	n/a	n/a	n/a	n/a	n/a	n/a	n/a		n/a	n/a	n/a	n/a		

↑ CIMT (≥75th centile)	n/a	90th	75th-90th	75th-90th	n/a	n/a	n/a	75th	n/a	>95th	>95th	75th	75th-90th	75th-90th
↓ FMD (<7.7%)	n/a	-	yes		n/a	n/a	n/a		n/a	Yes	Yes			Yes
Salivary outcomes														
Mean cortisol nmol/L	43.5	16.2	5.2	10.7	9.7	7.5	-	11.8	2.7	10.5	30.1	7.1	5.1	9.1
Mean cortisone nmol/L	17.9	9.1	6.7	5.8	6.3	12.4	-	14.4	11.4	21.1	17.5	10.0	11.1	16.1
Mean testosterone pmol/L	-	28	-	34.9	-	4.0	-	63.8	-	-	1404.9	61.5	176.8	179.4
Mean A4 pmol/L	-	71.9	-	131.9	-	32.3	-	86.8	-	-	4730.8	345.0	2419.0	112.1
Mean 11KT pmol/L	-	9.2	-	95.4	-	60.3	-	<6.0	-	-	84.7	401.5	3283.0	<6.0
Mean 11OHA4 pmol/L	-	40.0	-	94.7	-	84.3	-	109.5	-	-	1132.4	369.8	4809.2	1417.5
Mean 17OHP pmol/L	-	432.3	-	704.4	-	351.1	-	-	-	-	828.1	1301.5	12812.6	51.4

Abbreviations – ID: identifier; WB: White British; EE: Eastern European; A: Asian; Mi: mixed race; M: male; F: female; 21OHD: 21 hydroxylase deficiency; 3βHSD: 3βhydroxysteroid deficiency; GDM: gestational diabetes: Dex: dexamethasone; CVD: cardiovascular disease; FDR: first-degree relative; FH: familial hypercholesterolaemia; FHx: family history; mg/m^2/day: milligrams per meter squared per day; mcg/m^2/day: micrograms per metre squared per day; mmol/L: millimoles per litre; VWF: won Willebrand antigen and activity; BMI SDS: body mass index standard deviation score; CGM: continuous glucose monitoring system; HTN: hypertension; CIMT: carotid intima-media thickness; FMD: flow-mediated dilatation; nmol/L: nanomoles per litre; A4: androstenedione; pmol/L: picomoles per litre; 11KT: 11ketotestosterone; 11OHA4: 11βhydroxyandrostenedione; 17OHP: 17 hydroxyprogesterone

Table 9.2: Table summarising patients with simple virilising CAH

Study ID	003	005	016	017	020	022
Baseline characteristics						
Age (years)	16	11	12	12	14	3
Sex	F	M	M	M	F	F
Genotype	21OHD	21OHD	21OHD	21OHD	21OHD	21OHD
Ethnicity	WC	EE	WC	WC	WC	WC
Relevant history						
Antenatal history	-	-	-	-	-	-
Smoking history	-	-	-	Parental	-	Parental
CVD in FDR	-	-	-	-	-	
FH/participant ↑ lipid	-	-	-	-	-	Yes
Other			ASD/ODD		-	
Hydrocortisone (mg/m^2/day)	7.8	15.7	6.8	10.3	7.8	8.8
Glucose parameters						
Mean glucose (mmol/L)	6.4	6.3	6.4	6.0	7.0	5.2
% glucose<3mmol/L	0.0	0.0	0.0	0.0	0.0	0.0
% glucose>10mmol/L	1.1	0.0	1.7	0.0	0.5	0.0
Cardiovascular outcomes						
Insulin resistance			>97th			
↑ VWF antigen and activity						TNP
↑ Leptin (≥75th centile)		75th-95th	>97.5th	75th-95th		
BMI SDS	-0.7	1.6	3.7	2.0	0.7	1.5
↑ Renin						
Clinic HTN (≥95th centile)						
ABPM HTN (≥95th centile)			Yes	-		n/a
↑ cIMT (≥75th centile)	90th	>95th	>95th	75th	75th	n/a
↓ FMD (<7.7%)		-		Yes		n/a
Salivary outcomes						
Mean cortisol nmol/L	14.2	12.9	3.1	2.8	15.8	16.1
Mean cortisone nmol/L	14.1	9.9	18.9	11.6	13.6	19.3
Mean testosterone pmol/L	50.0	41.6	182.9	167.0	67.3	-
Mean A4 pmol/L	701.5	192.7	419.3	297.6	965.8	-
Mean 11KT pmol/L	468.5	91.7	128.4	419.7	603.1	-
Mean 11OHA4 pmol/L	626.7	90.3	200.3	230.8	623.3	-
Mean 17OHP (pmol/L)	1427.7	1806.7	124.3	894.9	1301.5	-

Abbreviations – ID: identifier; WB: White British; EE: Eastern European; A: asian; Mi: mixed race; M: male; F: female; 21OHD: 21 hydroxylase deficiency; 3βHSD: 3βhydroxysteroid deficiency; GDM: gestational diabetes: Dex: dexamethasone; CVD: cardiovascular disease; FDR: first-degree relative; FH: familial hypercholesterolaemia; FHx: family history; mg/m^2/day: milligrams per meter squared per day; mcg/m^2/day: micrograms per metre squared per day; mmol/L: millimoles per litre; VWF: won Willebrand antigen and activity; BMI SDS: body mass index standard deviation score; CGM: continuous glucose monitoring system; HTN: hypertension; CIMT: carotid intima-media thickness; FMD: flow-mediated dilatation; nmol/L: nanomoles per litre; A4: androstenedione; pmol/L: picomoles per litre; 11KT: 11ketotestosterone; 11OHA4: 11βhydroxyandrostenedione; 17OHP: 17 hydroxyprogesterone

Table 9.3: Table summarising patients with Addison's disease and primary AI of unknown aetiology

Study ID	**001**	**018**	**019**	**025**	**026**
Baseline characteristics					
Age (years)	15	12	16	13	18
Sex	F	M	M	M	M
Adrenal antibodies	Positive	Positive	Positive	Positive	Negative
Ethnicity	WC	M	WC	WC	WC
Relevant history					
Antenatal history	-	-	-	-	-
Smoking history	-	Parental	-	Parental	-
CVD in FDR	-	Stroke	-	-	-
FH/participant ↑ lipid	-	-	-	-	-
Hydrocortisone (mg/m^2/day)	22.5	14.3	10.3	25	10.3
Fludrocortisone (mcg/m^2/day)	100	107.1	102.6	90.9	88.2
Other		ASD/ADHD	Hypothyroid		
Glucose parameters					
Mean glucose (mmol/L)	6.1	7.0	6.0	5.5	6.4
% glucose<3mmol/L	0.0	0.2	0.1	0.0	0.0
% glucose>10mmol/L	0.0	0.0	0.1	0.0	0.1
Cardiovascular outcomes					
Insulin resistance	Yes	>97th		>97th	
VWF antigen and activity	Low			High	
↑ Leptin (≥85th centile)	75th-95th	>97.5th	75th-95th	>97.5th	
BMI SDS	1.9	1.4	1.0	2.5	0.52
↑ Renin	Yes		Yes		
Clinic HTN (≥95th centile)				Yes	
ABPM HTN (≥95th centile)					
↑ cIMT (≥75th centile)	75th	75th	75th	75th	
↓ FMD (<7.7%)			Yes		
Salivary outcomes					
Mean cortisol nmol/L	3.6	16.1	5.1	12.8	9.9
Mean cortisone nmol/L	11.0	11.8	11.3	17.9	21.8
Mean testosterone pmol/L	43.7	51.0	175.9	161.0	225.4
Mean A4 pmol/L	422.0	91.7	216.7	100.9	203.9
Mean 11KT pmol/L	<6.0	<6.0	<6.0	<6.0	<6.0
Mean 11OHA4 pmol/L	<45.0	92.4	<45.0	134.7	<45.0
Mean 17OHP pmol/L	50.8	20.1	34.1	27.1	77.0

Abbreviations – ID: identifier; WB: White British; EE: Eastern European; A: asian; Mi: mixed race; M: male; F: female; 21OHD: 21 hydroxylase deficiency; 3βHSD: 3βhydroxysteroid deficiency; GDM: gestational diabetes: Dex: dexamethasone; CVD: cardiovascular disease; FDR: first-degree relative; FH: familial hypercholesterolaemia; FHx: family history; mg/m^2/day: milligrams per meter squared per day; mcg/m^2/day: micrograms per metre squared per day; mmol/L: millimoles per litre; VWF: won Willebrand antigen and activity; BMI SDS: body mass index standard deviation score; CGM: continuous glucose monitoring system; HTN: hypertension; CIMT: carotid intima-media thickness; FMD: flow-mediated dilatation; nmol/L: nanomoles per litre; A4: androstenedione; pmol/L: picomoles per litre; 11KT: 11ketotestosterone; 11OHA4: 11βhydroxyandrostenedione; 17OHP: 17 hydroxyprogesterone

9.6. GRACE 1: Hydrocortisone doses, AGPs and salivary cortisol and cortisone profiles

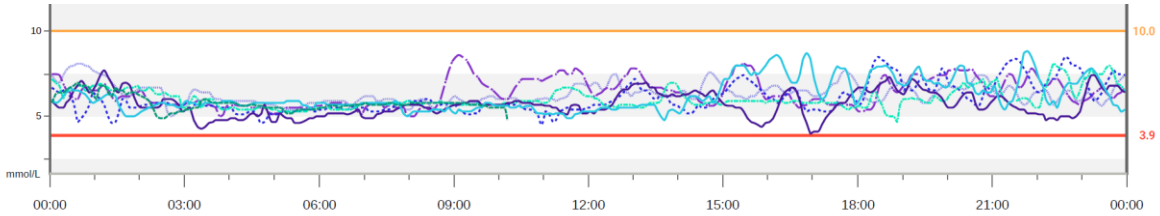
Patient 001

Hydrocortisone dose: 22.5mg/m2/day

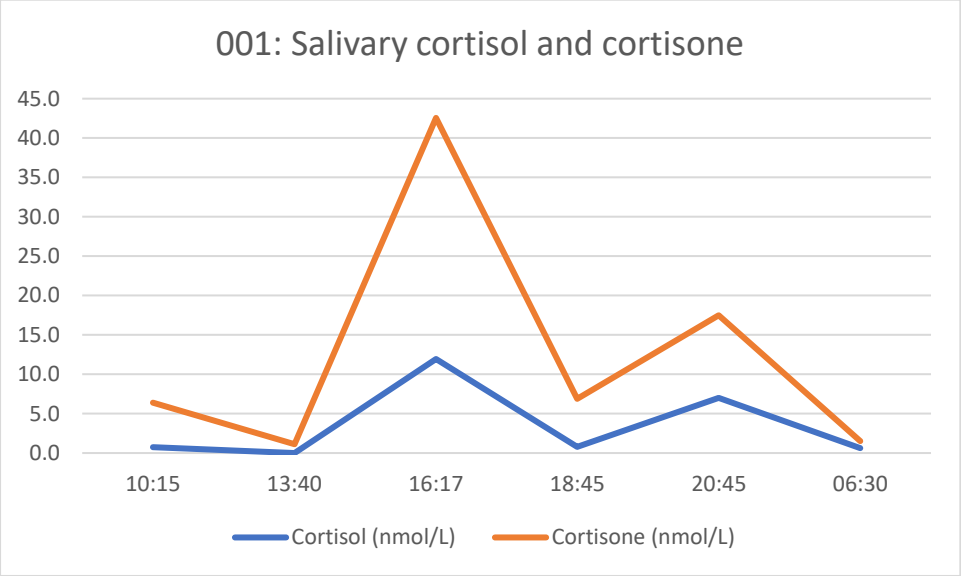
Hydrocortisone times: 20mg at 6am, 15mg at 13.30pm, 10mg at 6.30pm

Hydrocortisone formulation: standard

AGP:



Salivary profile:



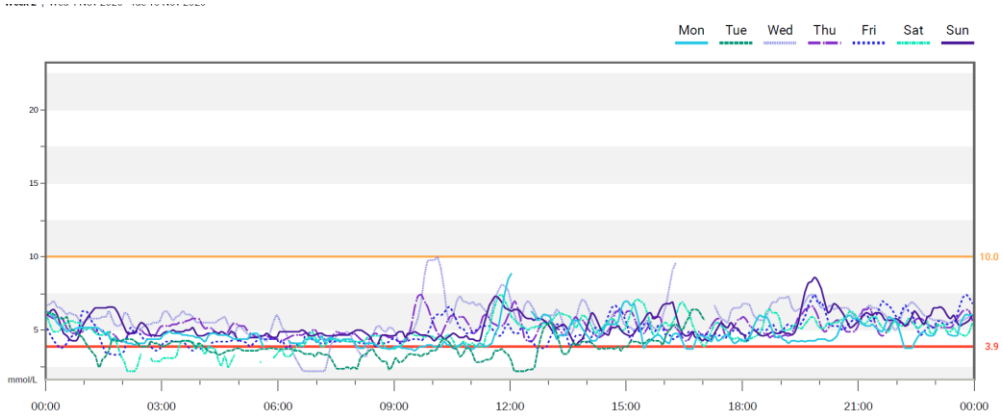
Patient 002

Hydrocortisone dose: 9.2mg/m2/day,

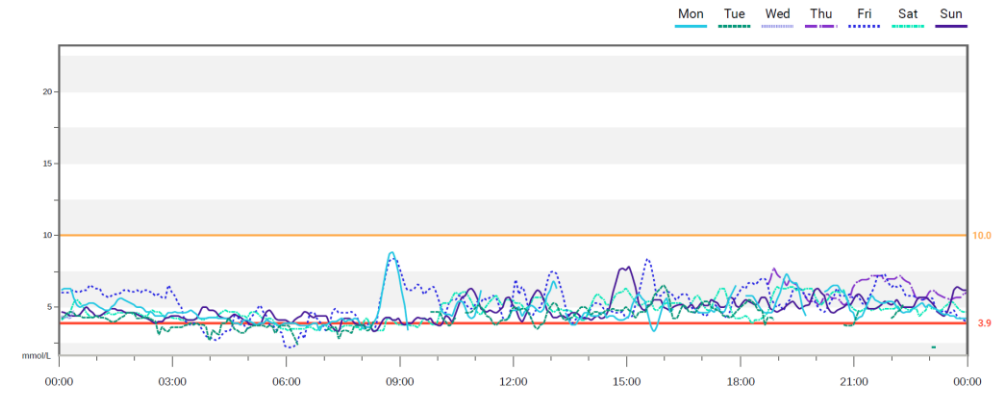
Hydrocortisone times: 1.5mg 1am, 2mg 7am, 1.5mg 12pm, 1.5mg 5pm

Hydrocortisone formulation: Standard

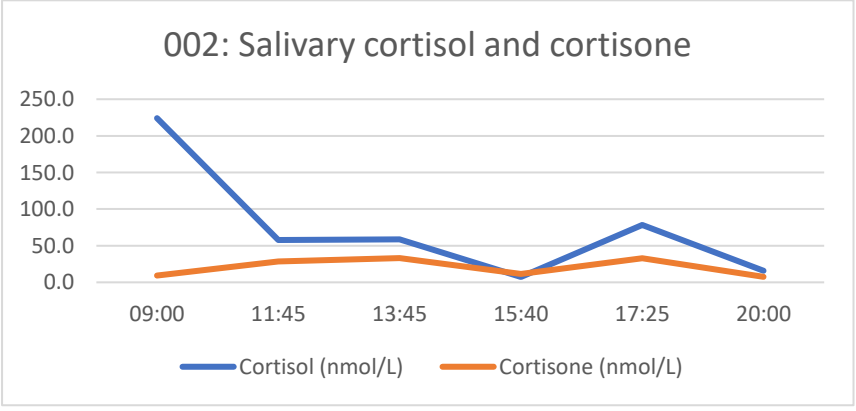
AGP:



AGP after manipulation of hydrocortisone:



Salivary profile:



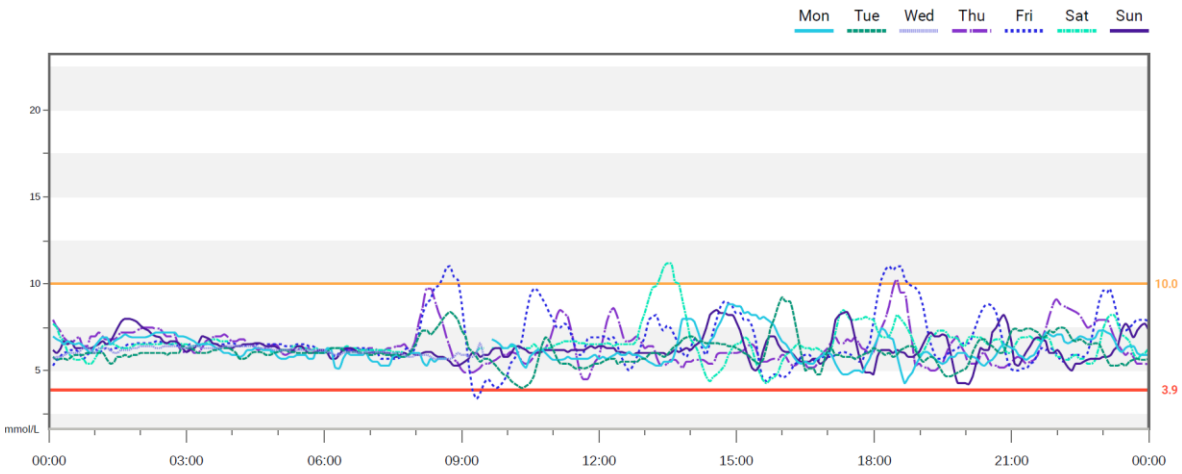
Patient 003

Hydrocortisone dose: 7.8mg/m2/day

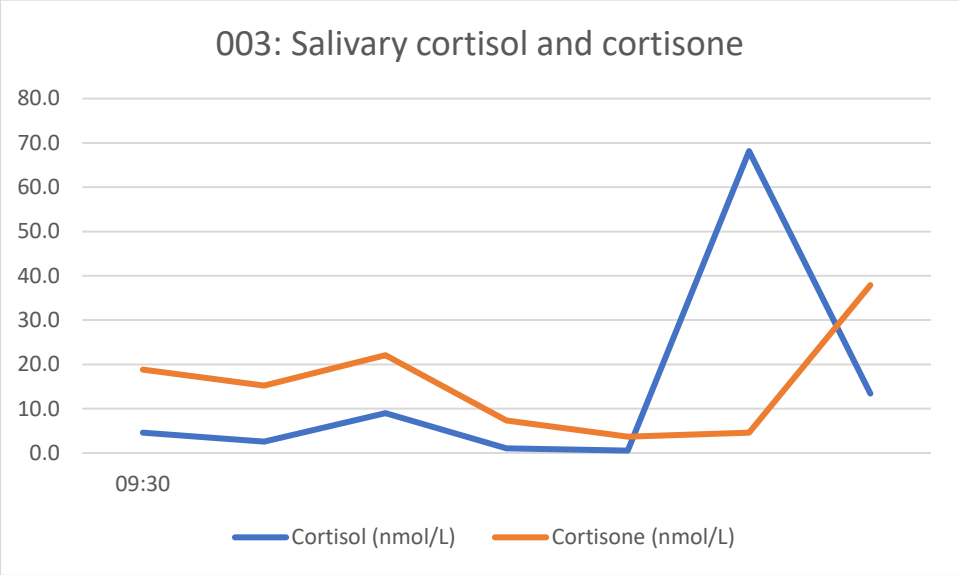
Hydrocortisone times: 7.5mg at 7am, 2.5mg at 2pm, 2.5mg at 10pm

Hydrocortisone formulation: standard

AGP:



Salivary profile:



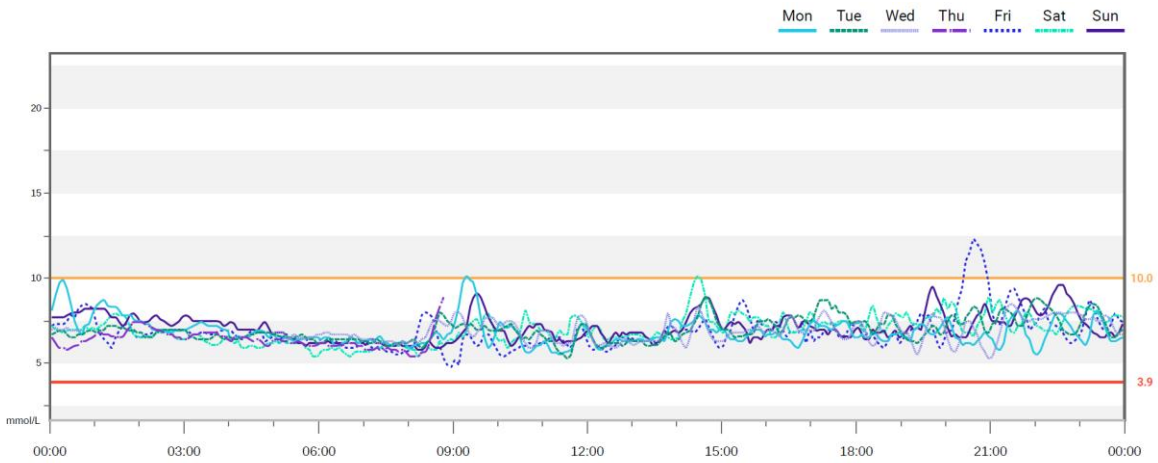
Patient 004

Hydrocortisone dose: 9.2mg/m2/day

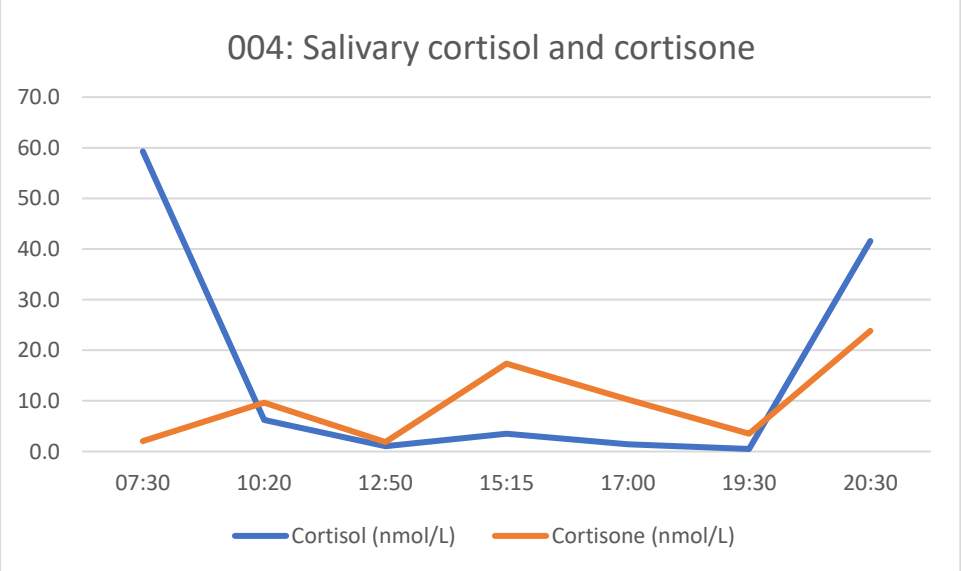
Hydrocortisone times: 2mg at 7am, 2mg at 1.30pm, 2mg at 7.15pm, 2mg at 10.20pm

Hydrocortisone formulation: standard

AGP:



Salivary profile:



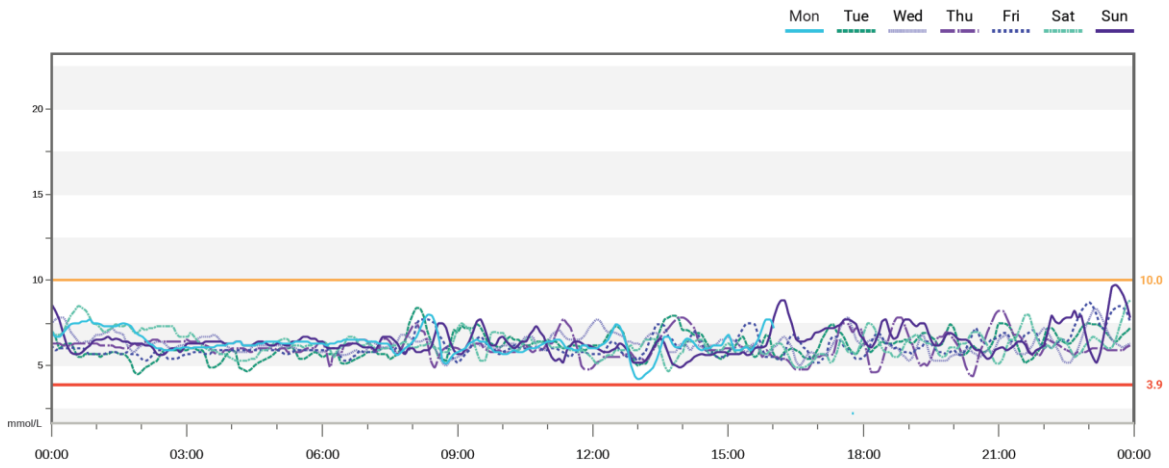
Patient 005

Hydrocortisone dose: 15.7 mg/m2/day

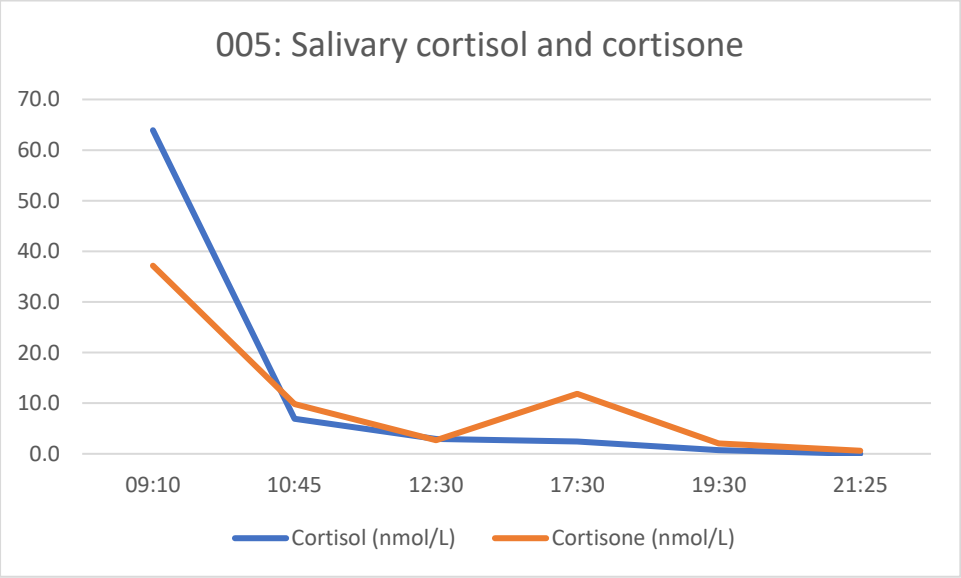
Hydrocortisone times: 12mg at 6am, 5mg at 1pm, 5mg Plenadren at 10pm

Hydrocortisone formulation: Standard, standard, Plenadren

AGP:



Salivary profile:



Patient 006

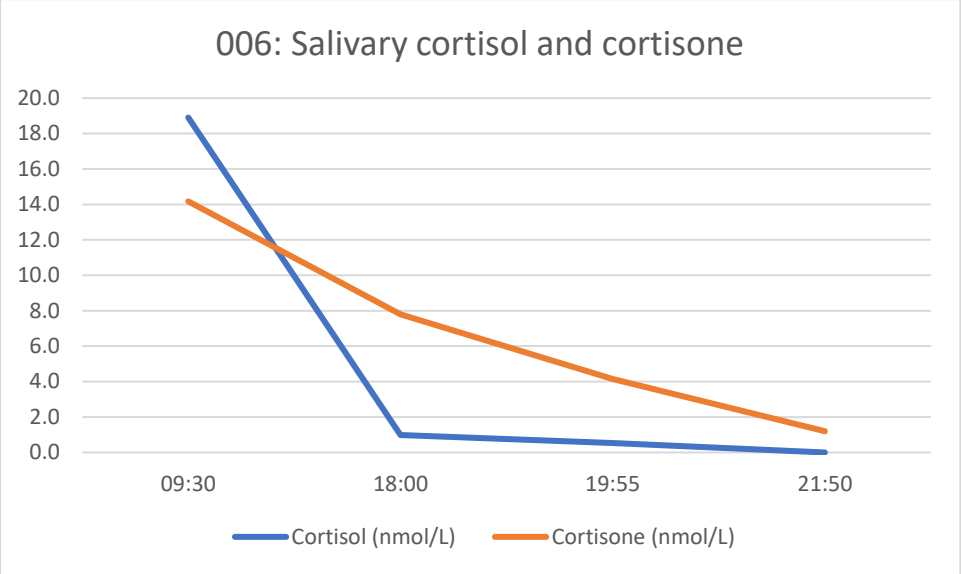
Hydrocortisone dose: 10.8mg/m2/day

Hydrocortisone times: 3mg at 7am, 2mg at 1pm, 2mg at 10pm

Hydrocortisone formulation: standard

AGP: Not worn

Salivary profile:



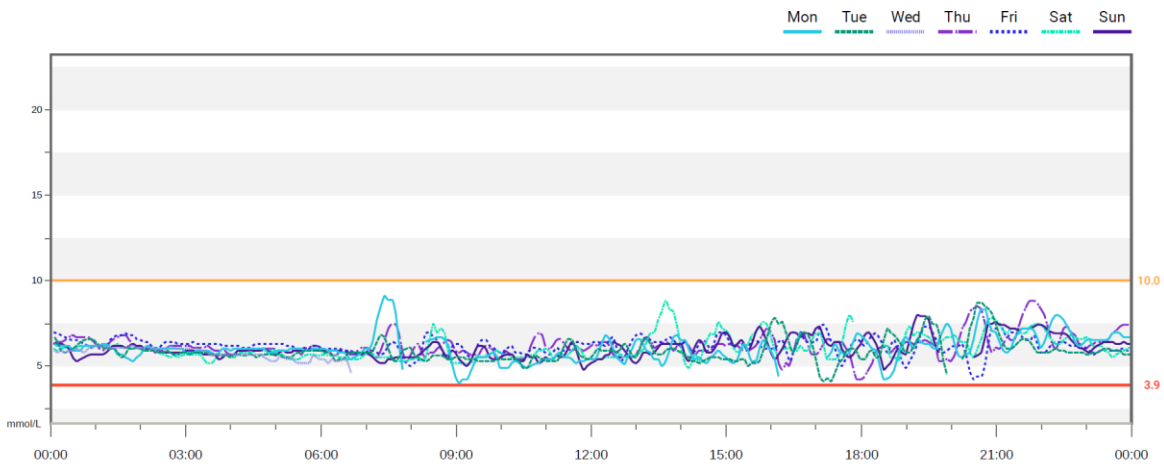
Patient 007

Hydrocortisone dose: 11.1mg/m2/day

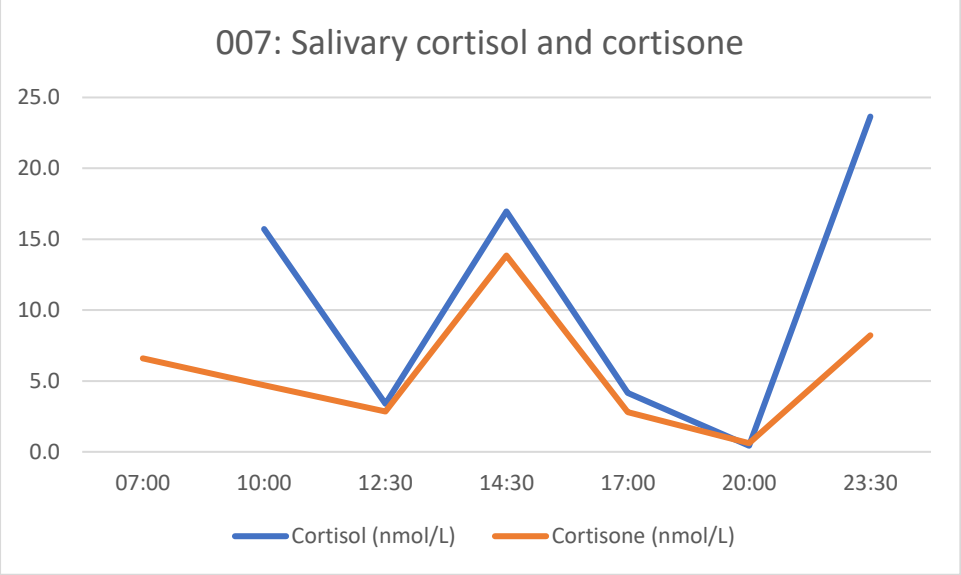
Hydrocortisone times:2mg at 6am, 2mg at 12pm, 2mg at 6pm, 2mg at midnight

Hydrocortisone formulation: standard

AGP:



Salivary profile:



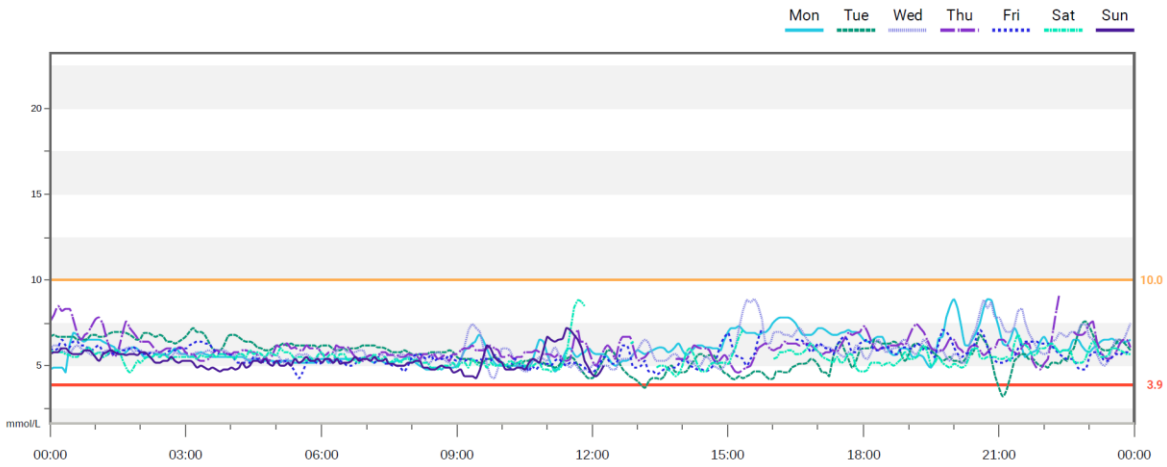
Patient 008

Hydrocortisone dose: 9.9mg/m2/day

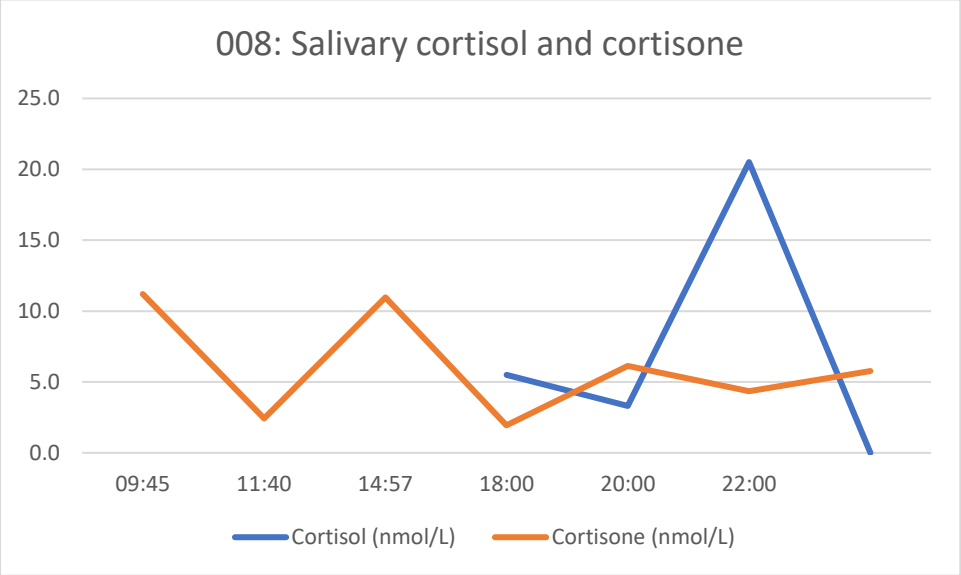
Hydrocortisone times: 2.5mg at 6am, 1.5mg at 12pm. 1.5mg at 6pm1.5mg at midnight

Hydrocortisone formulation: standard

AGP:



Salivary profile:



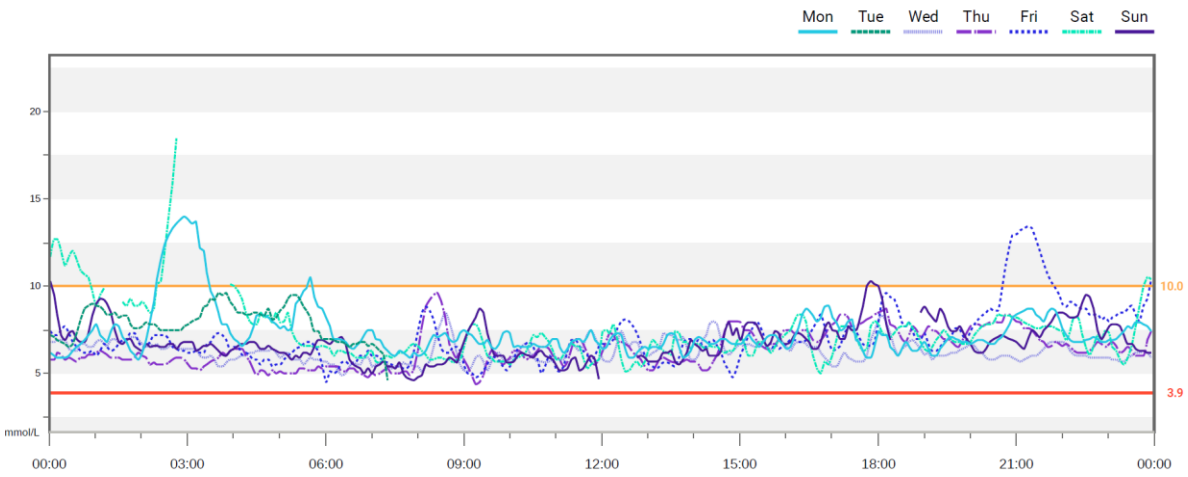
Patient 009

Hydrocortisone dose: 9.4mg/m2/day

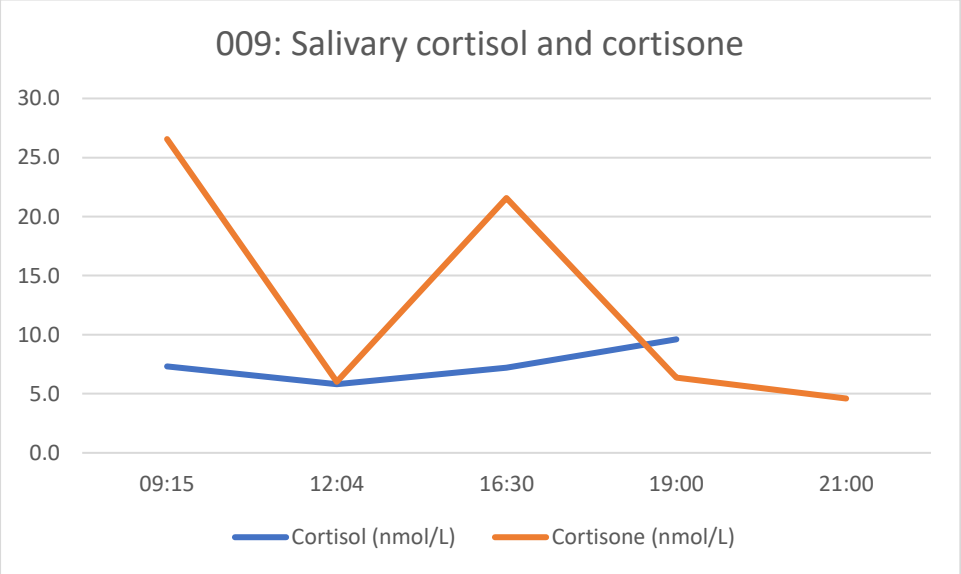
Hydrocortisone times: 2mg at 7am, 2mg at 2pm, 1.5mg at 8pm

Hydrocortisone formulation: standard

AGP:



Salivary profile:



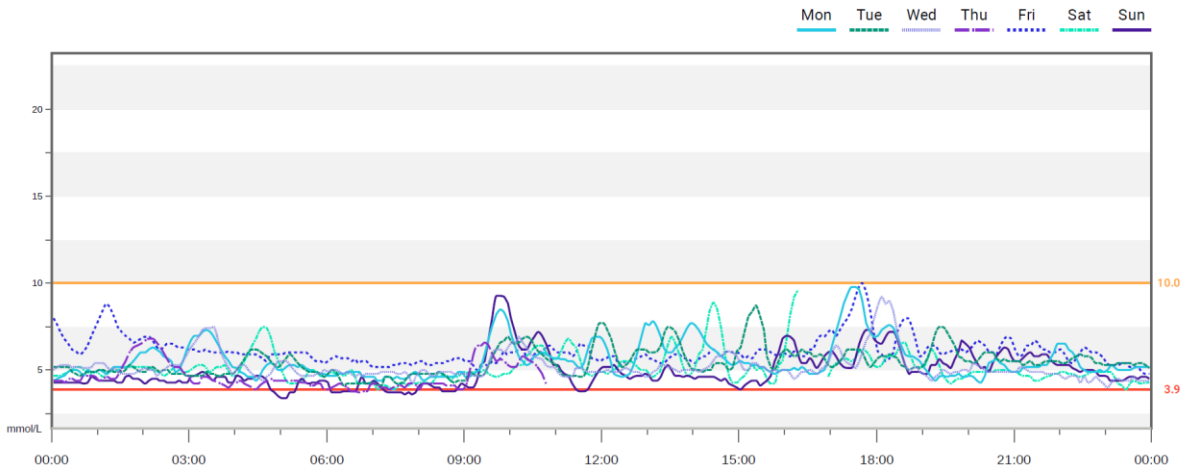
Patient 010

Hydrocortisone dose:9.8mg/m2/day

Hydrocortisone times: 2.5mg at 7am, 1mg at 12.30pm,1mg at 8pm, 1mg at 11pm

Hydrocortisone formulation: standard

AGP:



Salivary profile: not performed

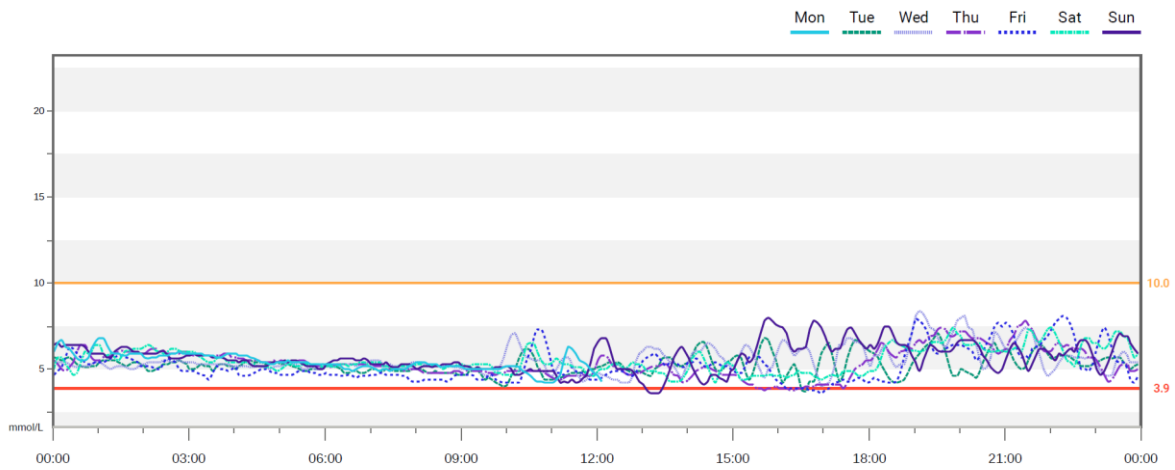
Patient 011

Hydrocortisone dose: 5.6mg/m2/day

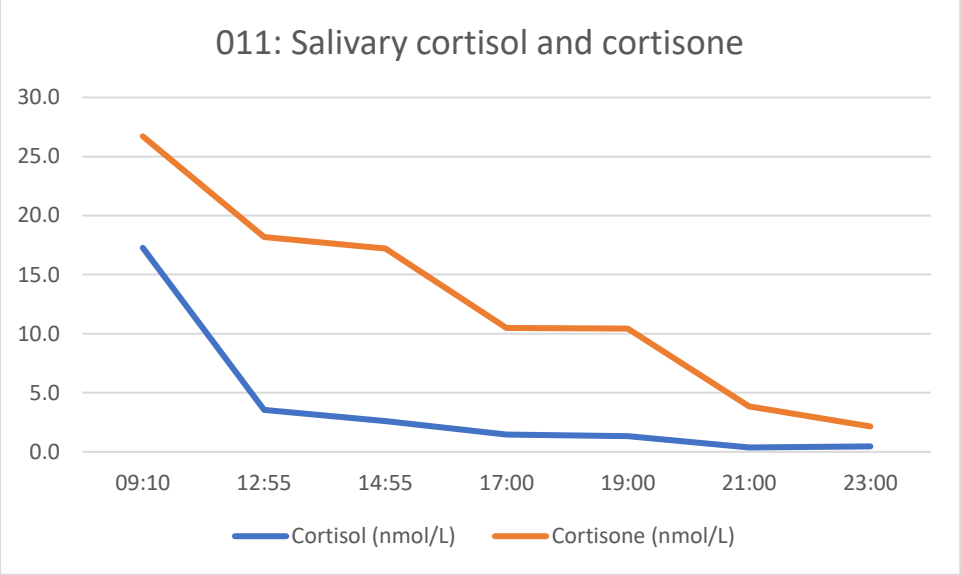
Hydrocortisone times: 3mg at 7am, 3mg at 12.30pm, 3mg at 8.45pm

Hydrocortisone formulation: standard

AGP:



Salivary profile:



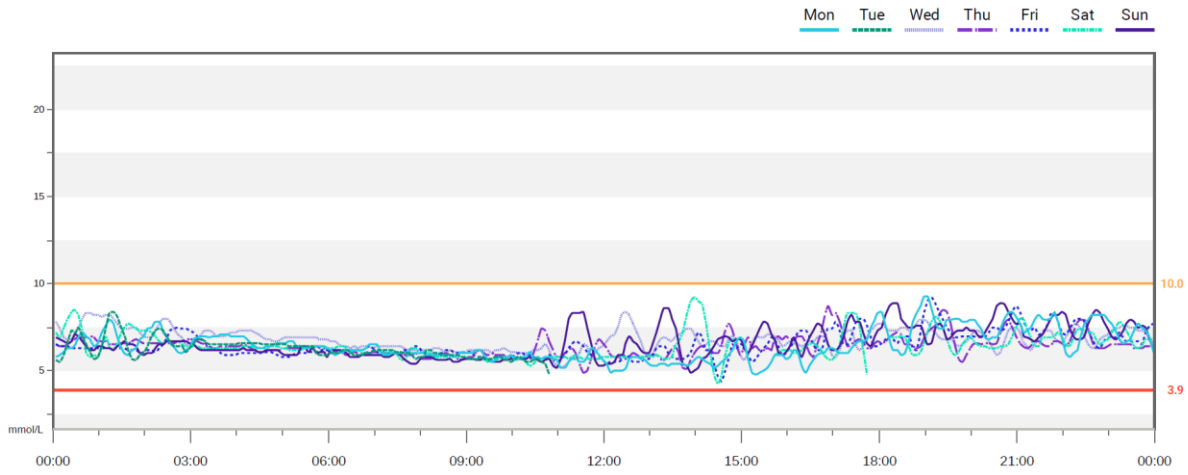
Patient 012

Hydrocortisone dose: 12.5mg/m2/day

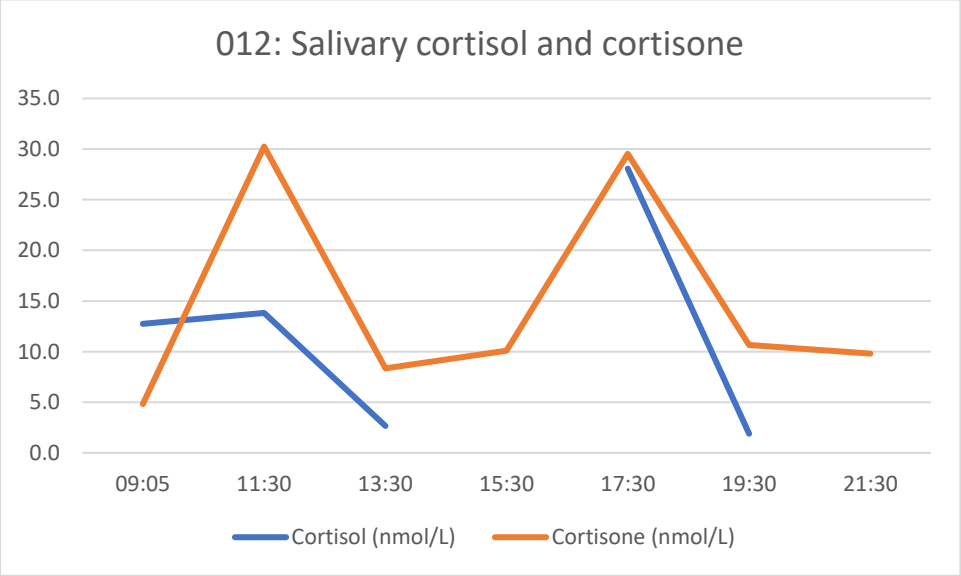
Hydrocortisone times:2.5mg at 3am, 5mg at 9.45am, 5mg at 2.45pm, 5mg at 9pm

Hydrocortisone formulation: standard

AGP:



Salivary profile:



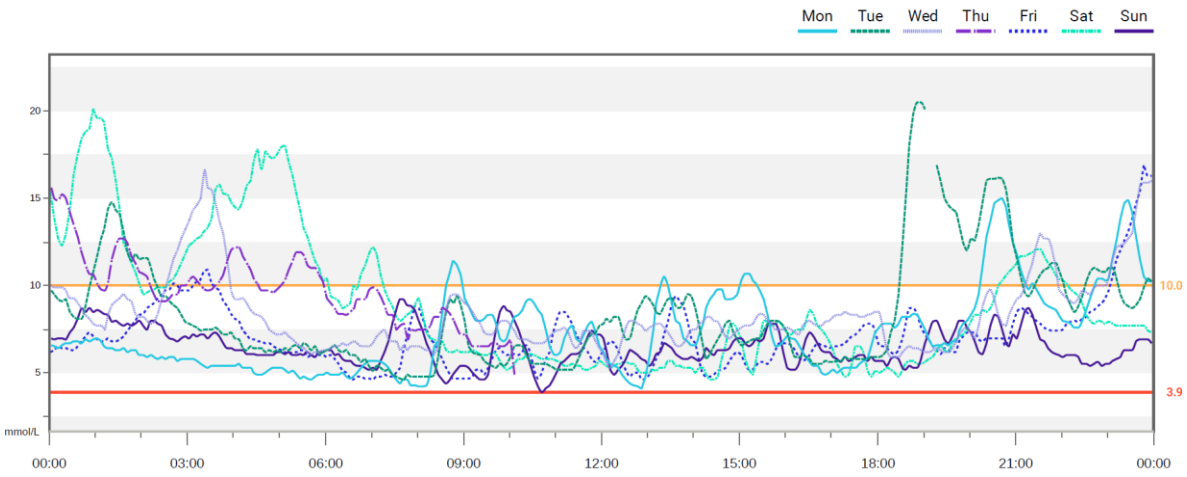
Patient 013

Hydrocortisone dose: 9.2mg/m2/day

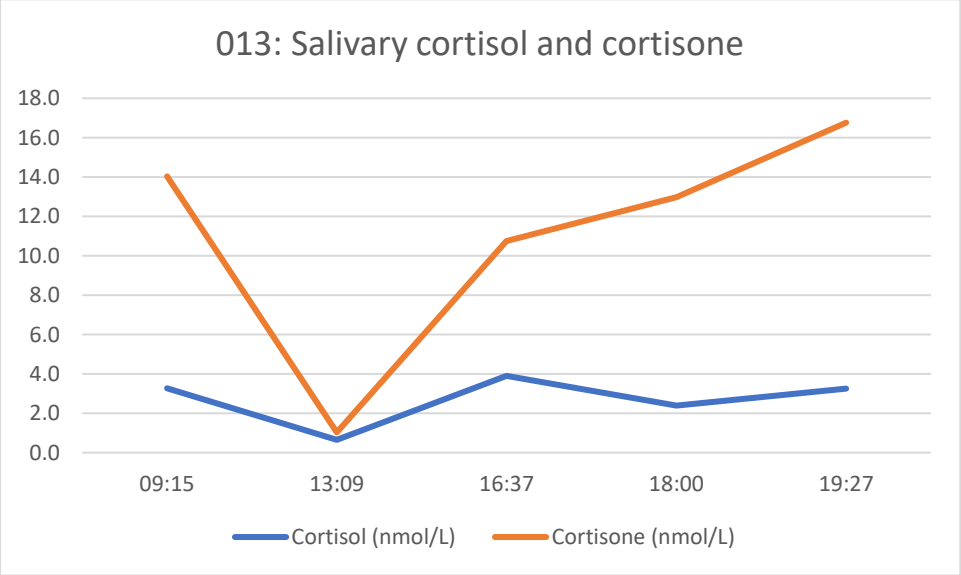
Hydrocortisone times: 2.5mg at 7am, 2mg at 1pm, 2mg at 7.30pm

Hydrocortisone formulation: standard

AGP:



Salivary profile:



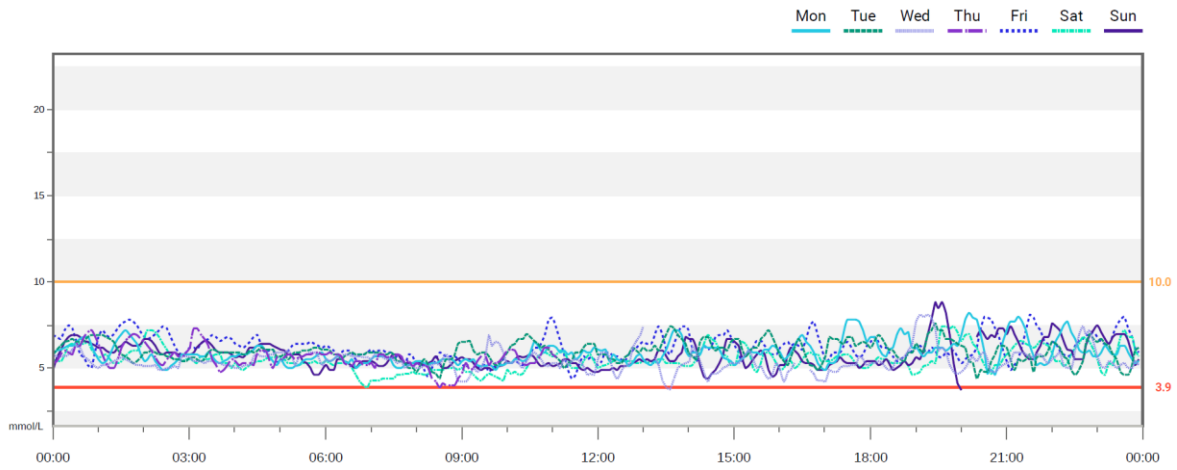
Patient 014

Hydrocortisone dose: 11.1mg/m2/day

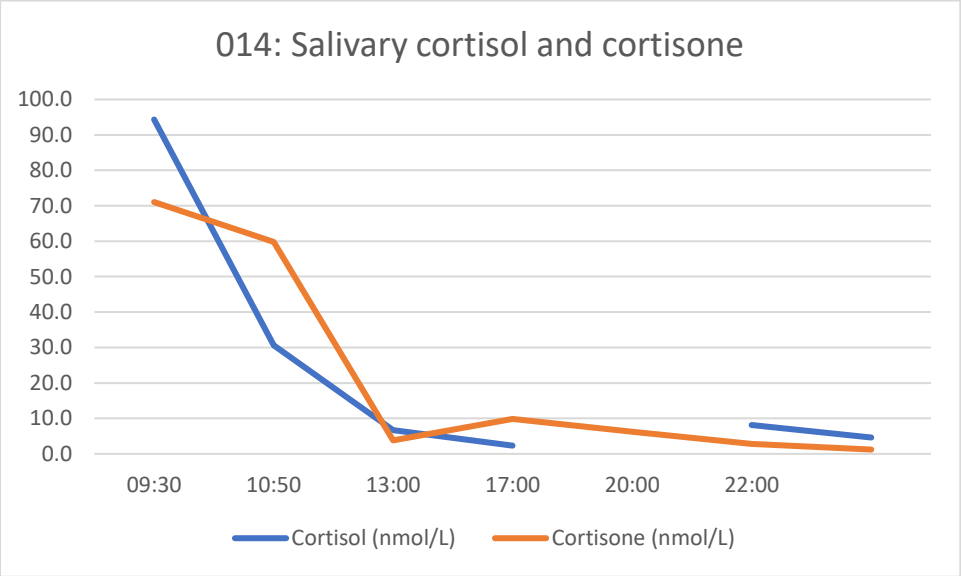
Hydrocortisone times: 4mg at 8.30am, 2.5mg at 4pm, 2.5mg at midnight

Hydrocortisone formulation: standard

AGP:



Salivary profile:



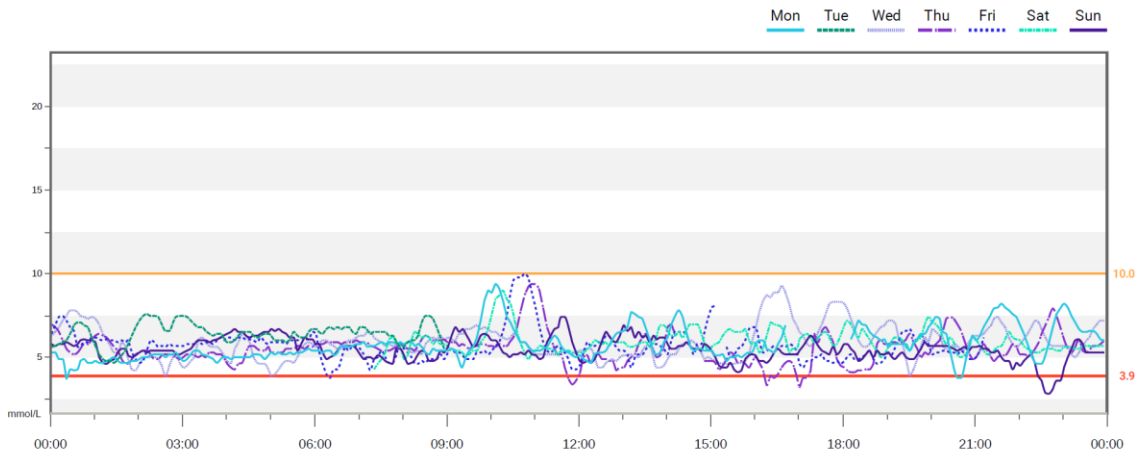
Patient 015

Hydrocortisone dose: 10.2mg/m2/day

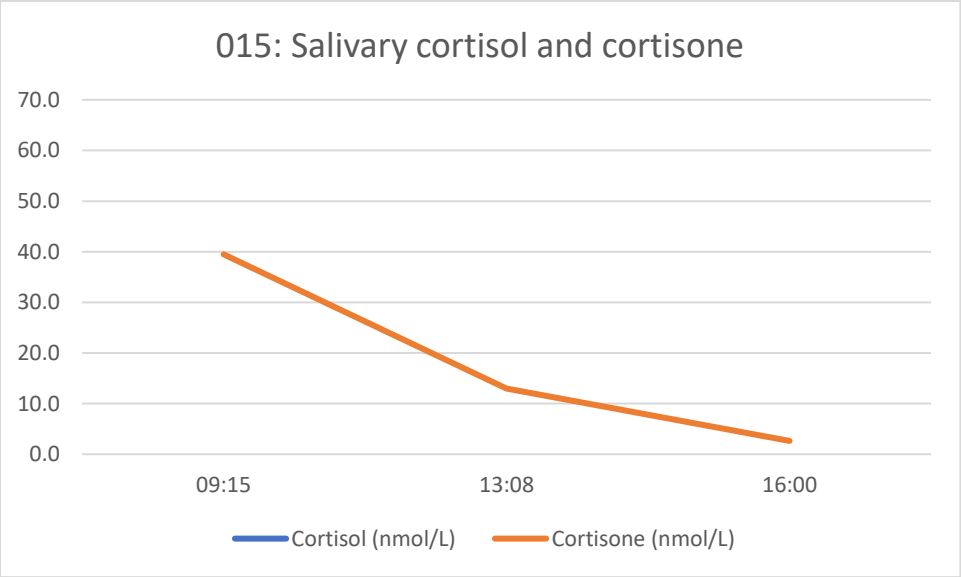
Hydrocortisone times: 3mg at 8am, 3mg at 2pm, 3mg at 7pm

Hydrocortisone formulation: standard

AGP:



Salivary profile:



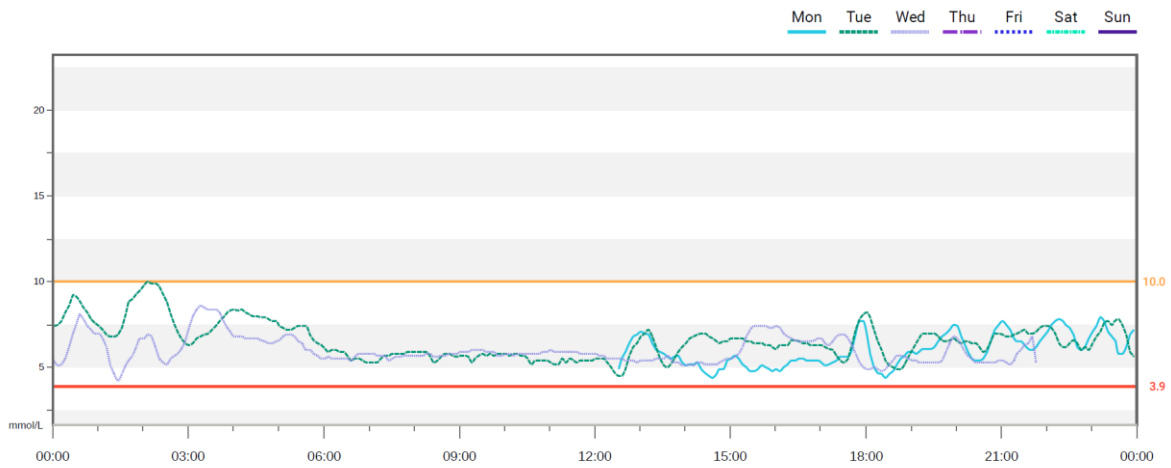
Patient 016

Hydrocortisone dose: 6.8mg/m2/day

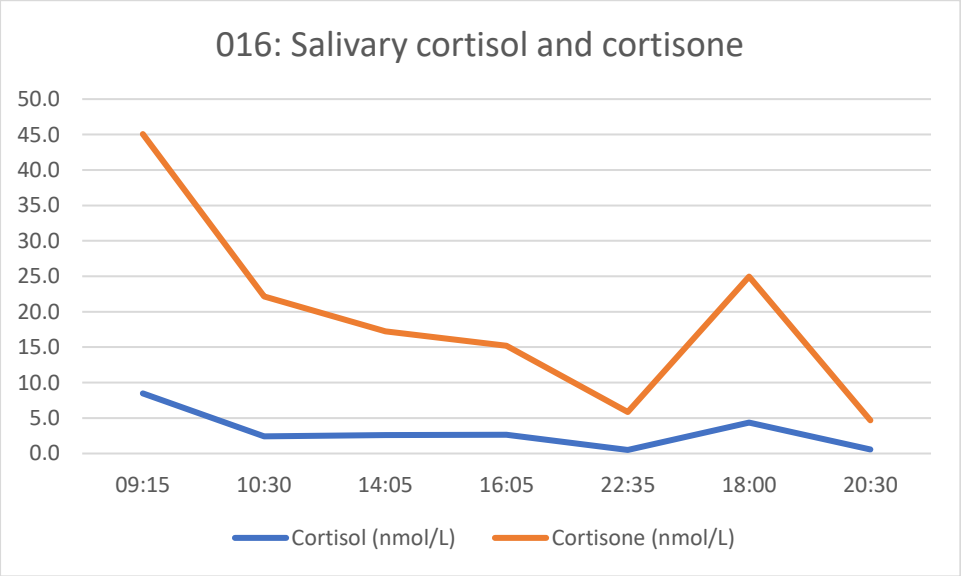
Hydrocortisone times: 5mg at 6.30am, 5mg at 11.30am, 5mg at 4pm

Hydrocortisone formulation: standard

AGP:



Salivary profile:



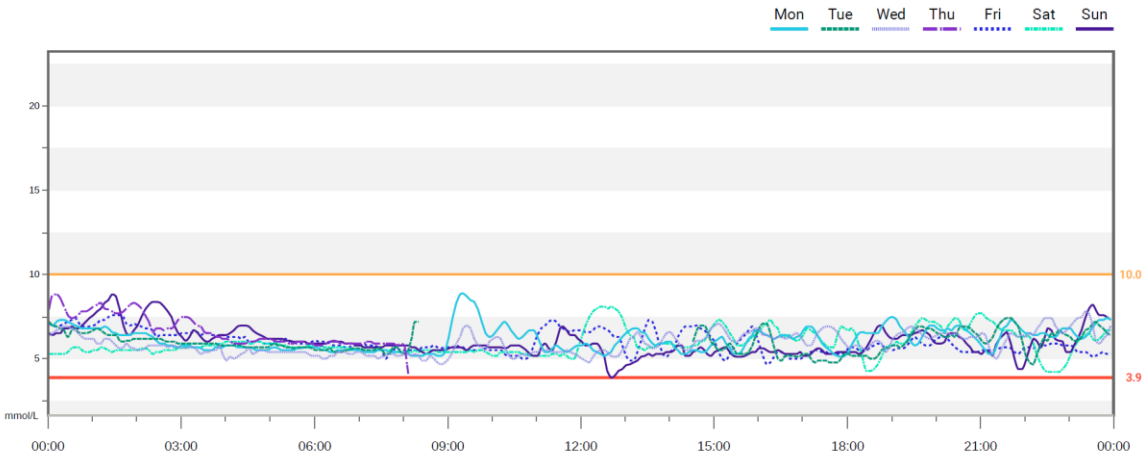
Patient 017

Hydrocortisone dose: 10.3mg/m2/day

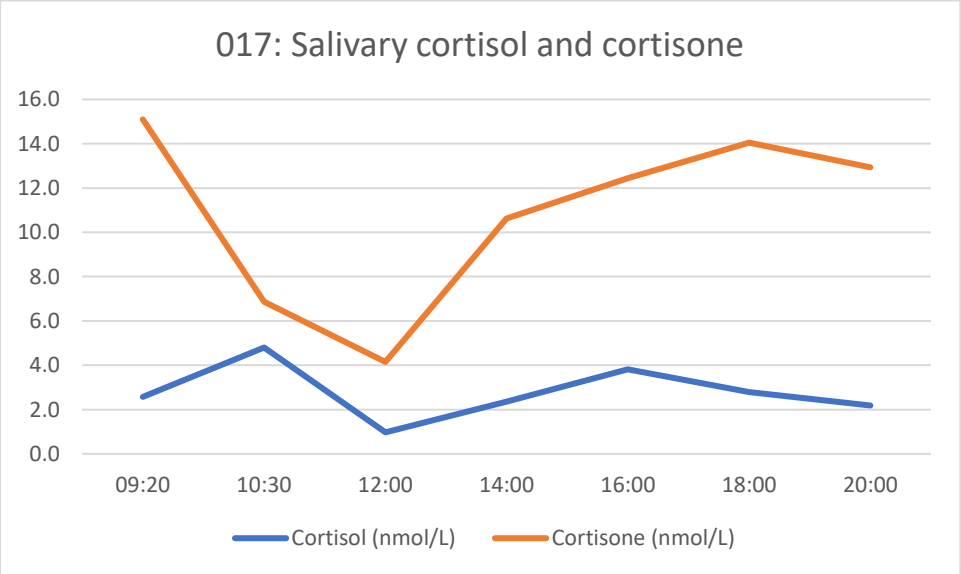
Hydrocortisone times:5mg at 7.30am, 5mg at 12pm, 2.5mg at 6p, 5mg at midnight

Hydrocortisone formulation: standard

AGP:



Salivary profile:



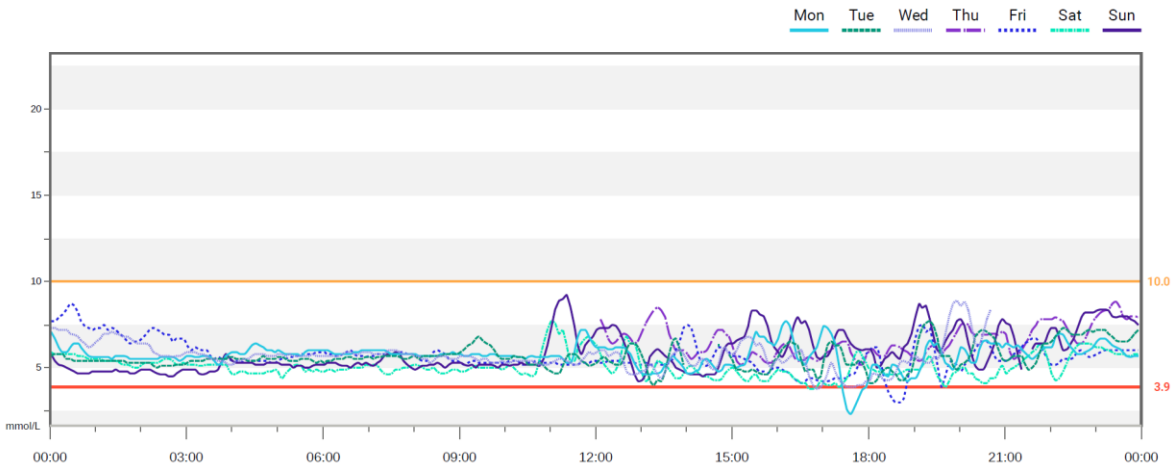
Patient 018

Hydrocortisone dose: 14.3mg/m2/day

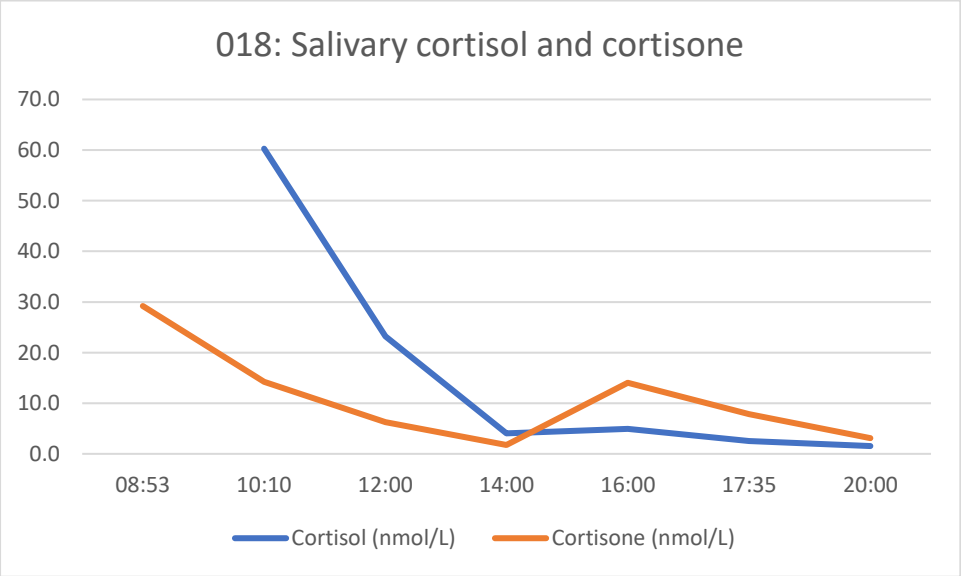
Hydrocortisone times: 5mg standard at 5am, 10mg Plenadren 8am, 10mg Plenadren 2pm

Hydrocortisone formulation: standard and Plenadren

AGP:



Salivary profile:



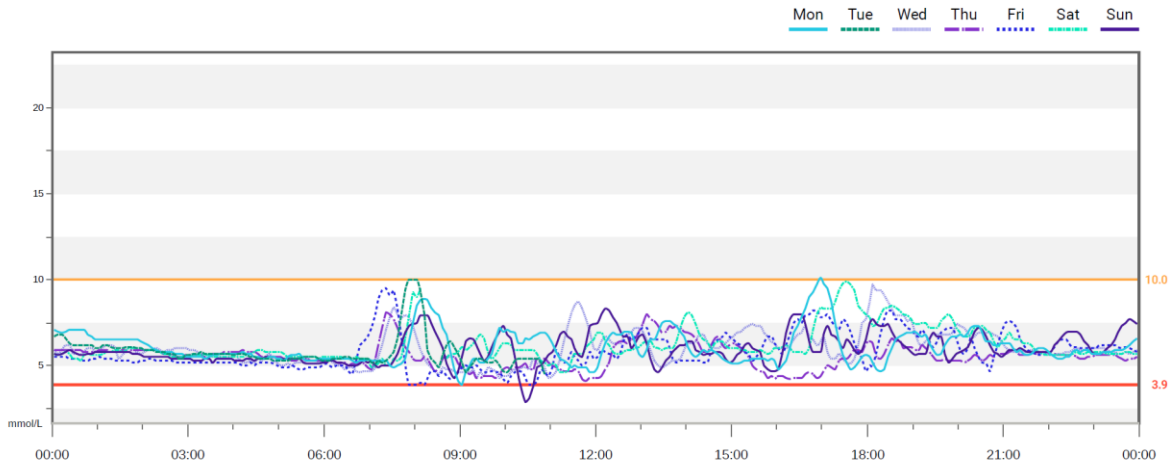
Patient 019

Hydrocortisone dose: 10.3mg/m2/day

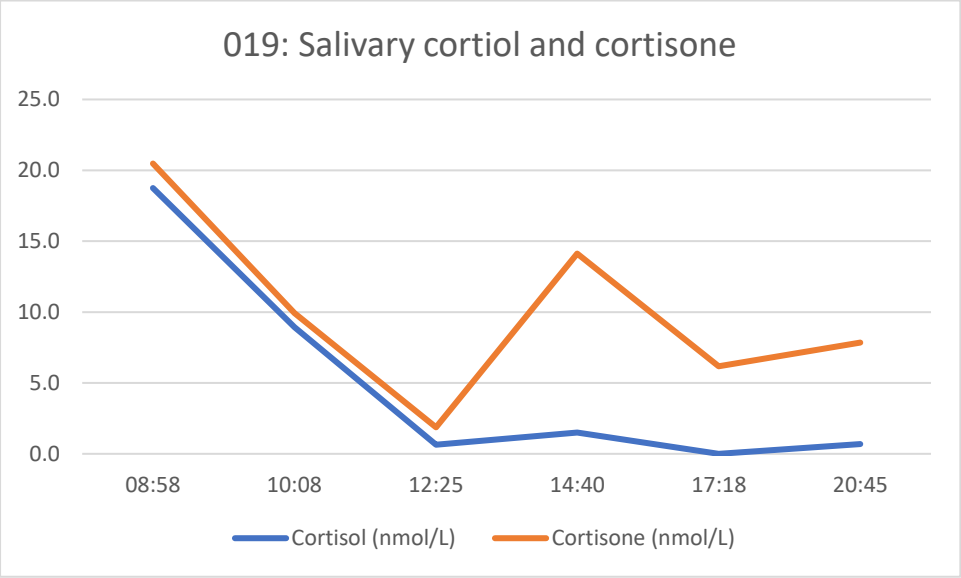
Hydrocortisone times: 10mg at 6.15am, 5mg at 12.30pm, 5mg at 5.30pm

Hydrocortisone formulation: standard

AGP:



Salivary profile:



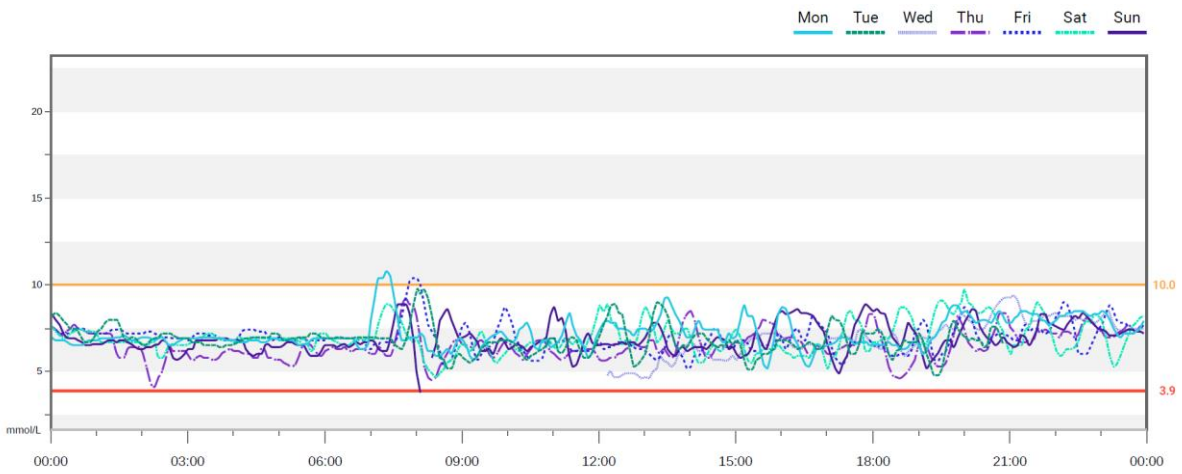
Patient 020

Hydrocortisone dose: 7.8mg/m2/day

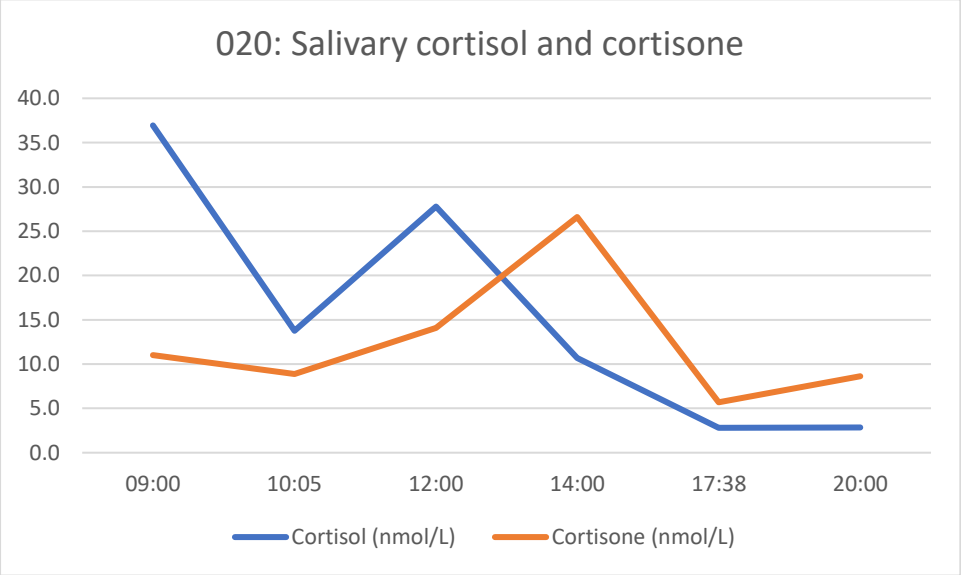
Hydrocortisone times: 1.5mg at 10.15am, 5mg at 3am

Hydrocortisone formulation: standard

AGP:



Salivary profile:



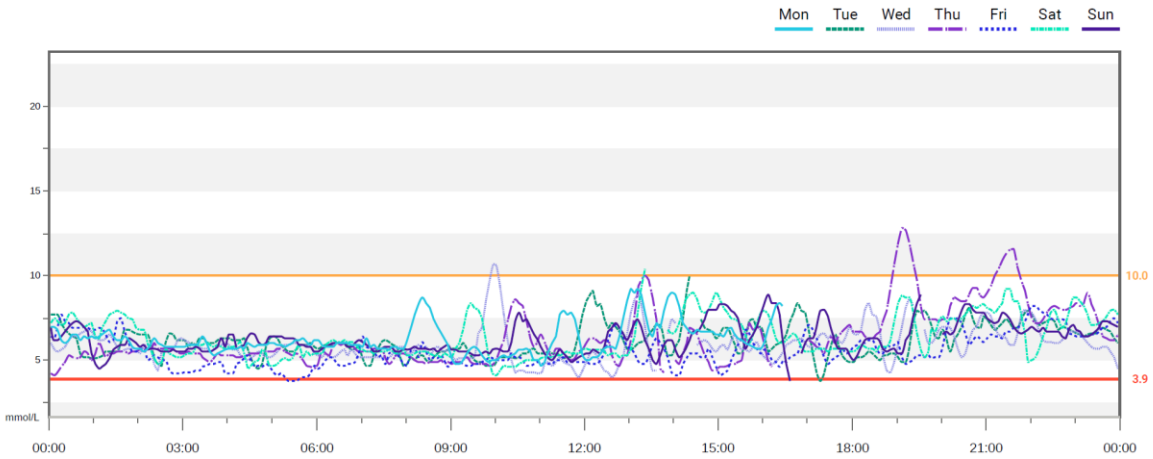
Patient 021

Hydrocortisone dose: 8.7mg/m2/day

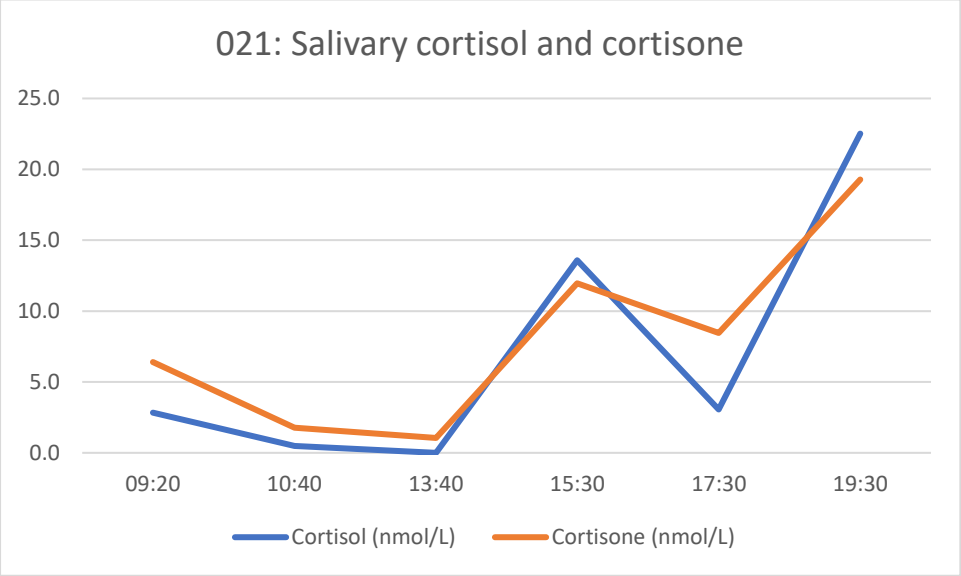
Hydrocortisone times: 3.5mg at 7am, 2.5mg at 2.30pm, 2.5mg at 7pm

Hydrocortisone formulation: standard

AGP:



Salivary profile:



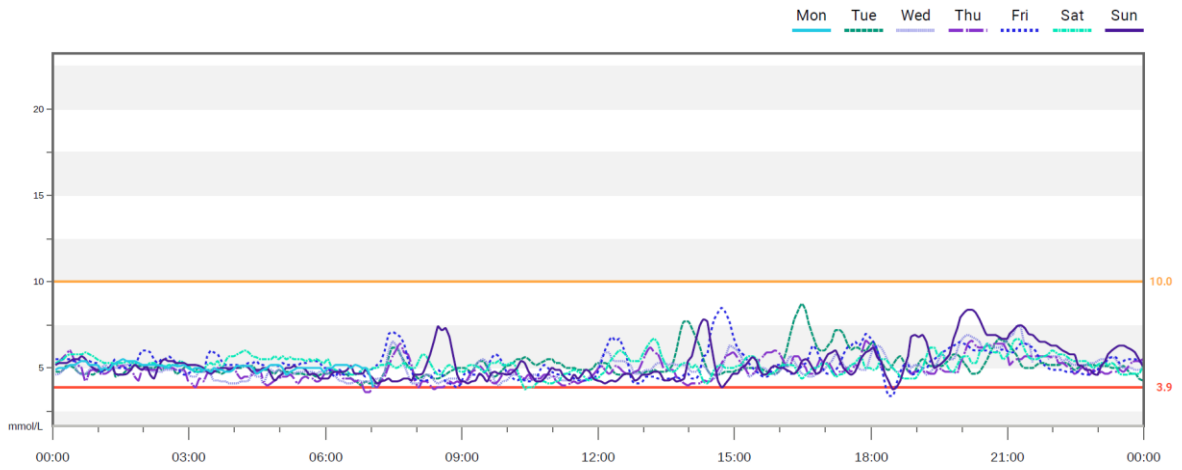
Patient 022

Hydrocortisone dose: 8.8mg/m2/day

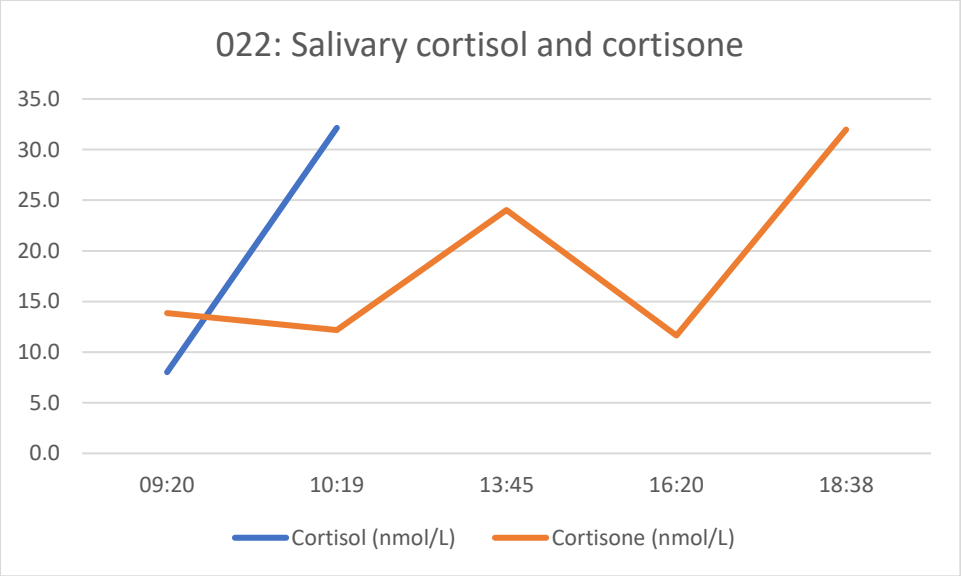
Hydrocortisone times: 2mg at 6am, 1.5mg at 12.30pm, 1.5 mg at 6pm, 1.5mg at 10pm

Hydrocortisone formulation: standard

AGP:



Salivary profile:



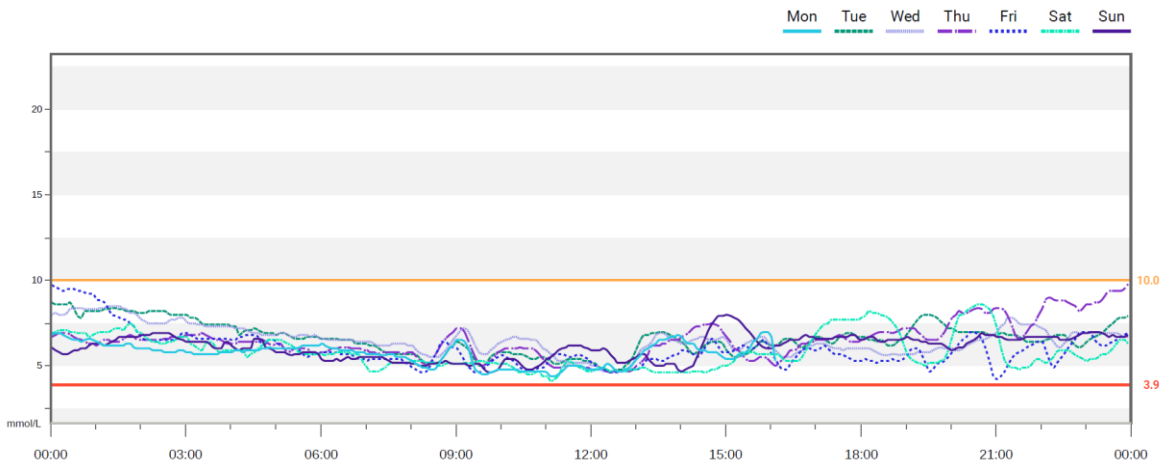
Patient 023

Hydrocortisone dose: 9.5mg/m2/day

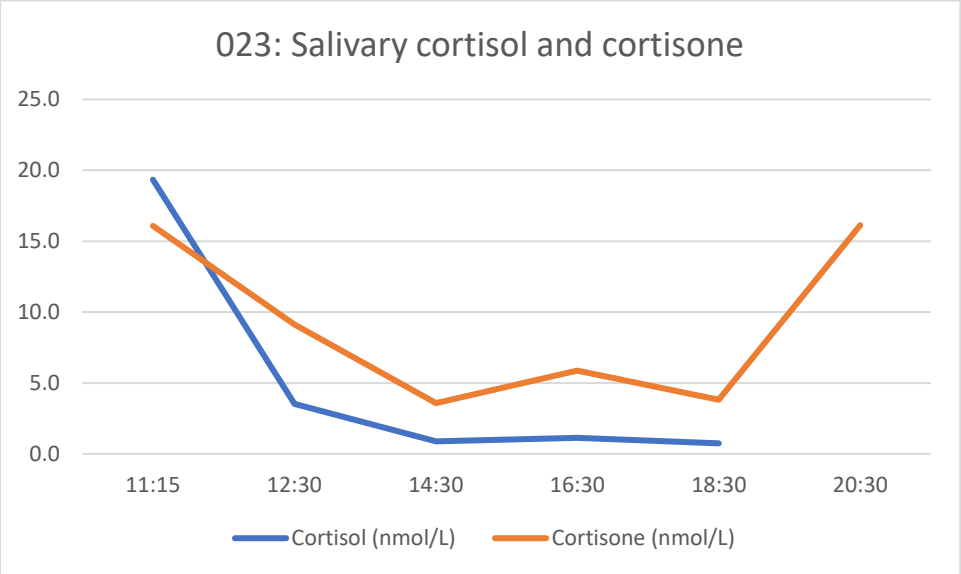
Hydrocortisone times: 10mg at 8am, 5mg at 3pm, 5mg at 10pm

Hydrocortisone formulation: standard

AGP:



Salivary profile:



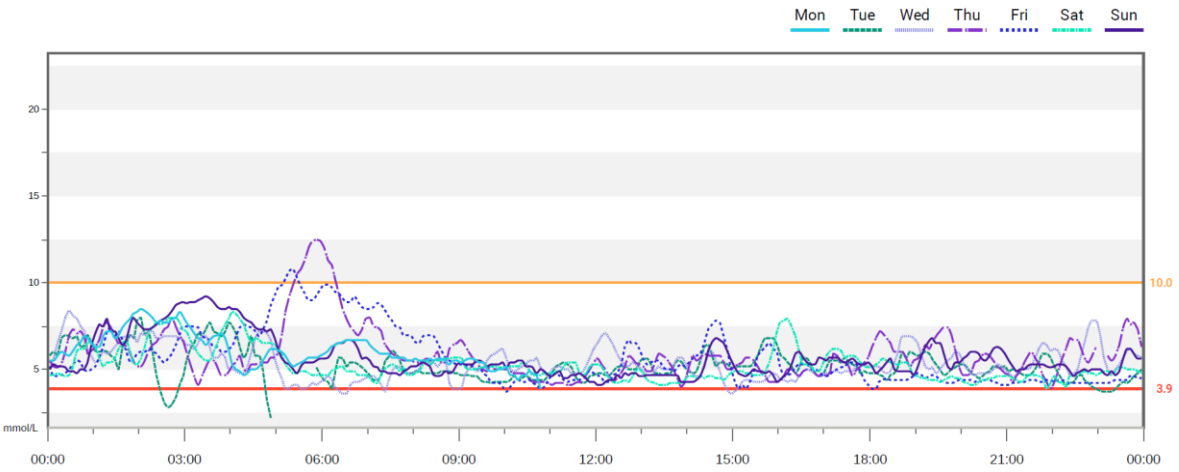
Patient 024

Hydrocortisone dose: 9.1mg/m2/day

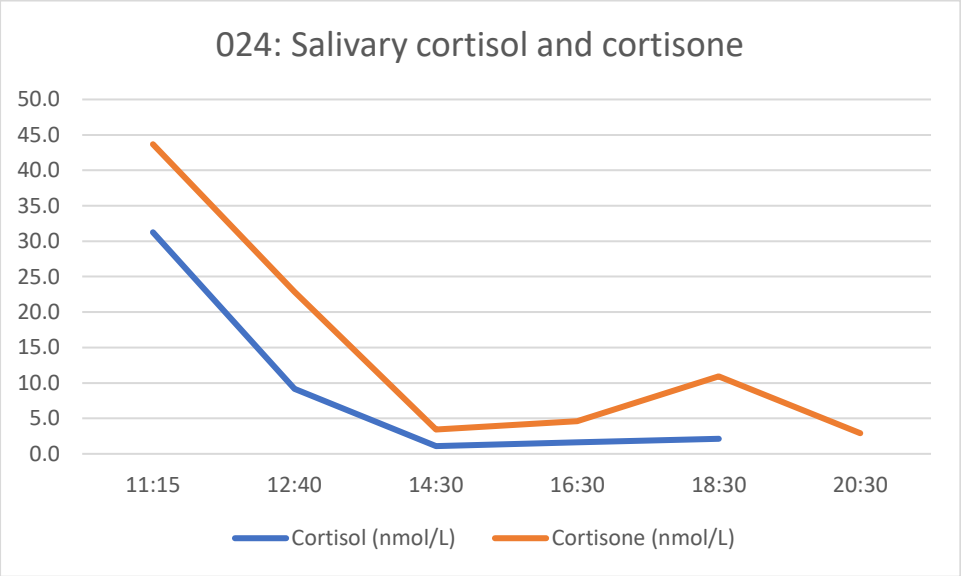
Hydrocortisone times:10mg at 8am, 5mg at 4pm, 5mg at 10pm

Hydrocortisone formulation: standard

AGP:



Salivary profile:



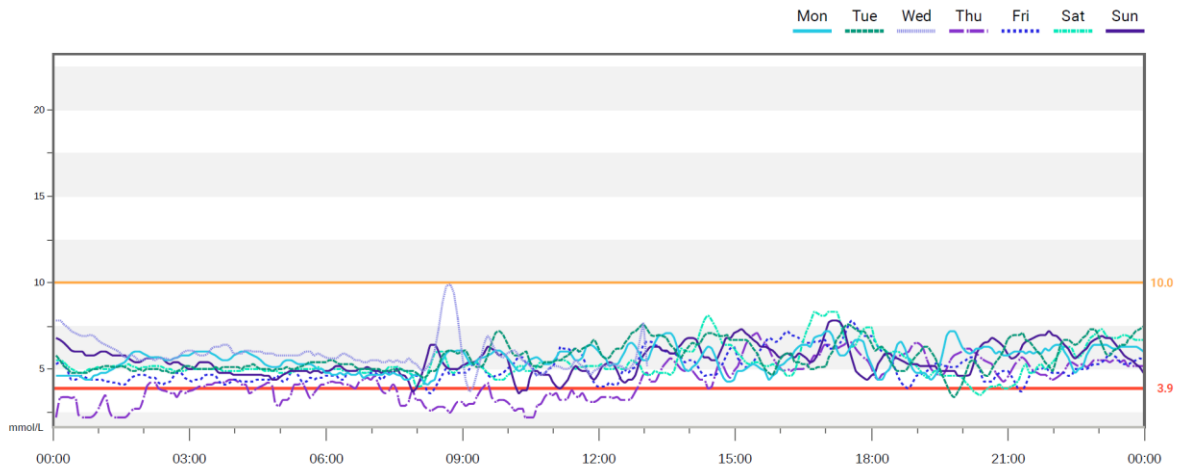
Patient 025

Hydrocortisone dose: 25mg/m2/day

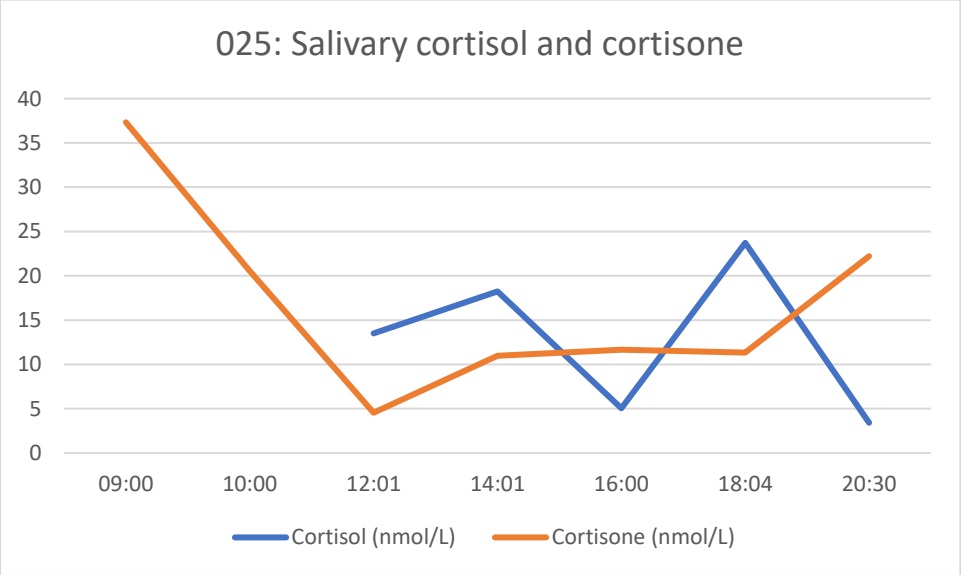
Hydrocortisone times: 10mg standard and 30mg Plenadren at 7am, 15mg Plenadren at 12pm, 10mg Plenadren at 10mg

Hydrocortisone formulation: combination of Plenadren and standard

AGP:



Salivary profile:



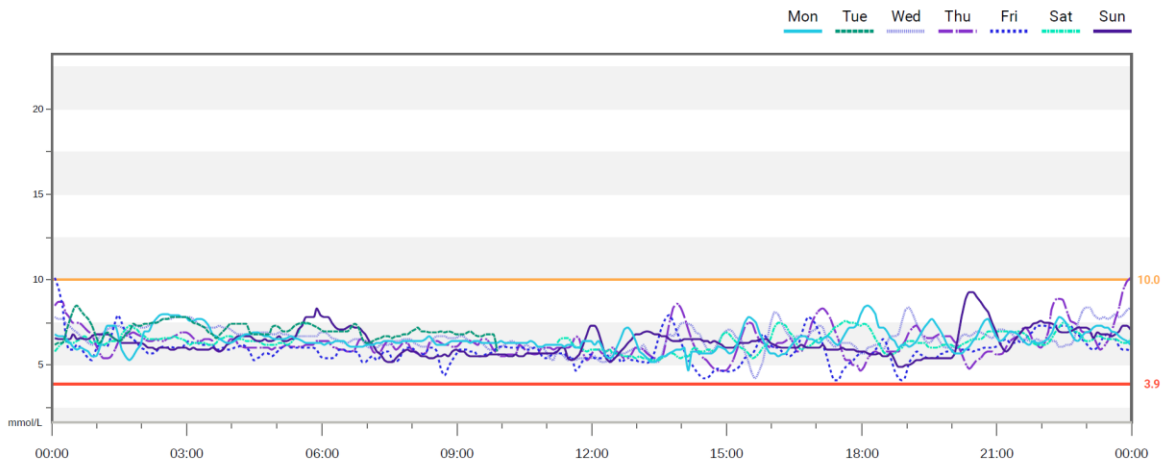
Patient 026

Hydrocortisone dose: 10.3mg/m2/day

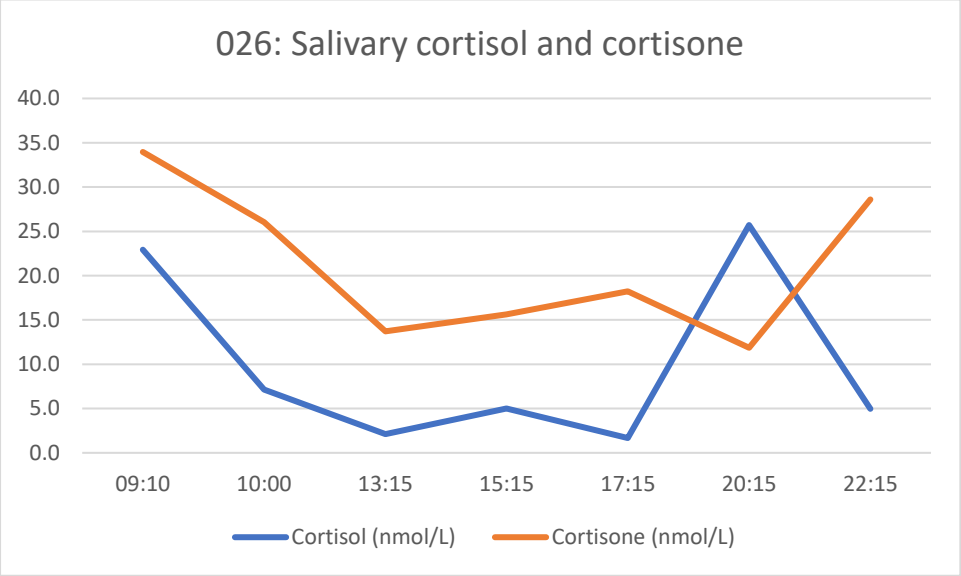
Hydrocortisone times: 5mg at 7.30am, 5mg at 1pm, 5mg at 7pm, 5mg 11pm

Hydrocortisone formulation: standard

AGP:



Salivary profile:



9.7. Example of participant information leaflet and consent form (parent version) for GRACE

Glucose Regulation and Cardiovascular Health in Adrenal Insufficiency (part 2)

PARENT INFORMATION LEAFLET AND CONSENT FORM

Version 1.0 9th February 2022

You and your child are being invited to take part in a research study. Before deciding whether you wish to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Ask us if there is anything that is not clear or if you would like more information.

What is this study about?

This study includes children and young people who have a diagnosis of adrenal insufficiency. This study is designed to find out whether children and young people are showing effects of over/under treatment of their adrenal disease.

We are looking at salivary hormone levels prior to the first hydrocortisone dose in the morning and a further one two hours later, to assess whether we can use this to guide treatment. It will be compared with a healthy cohort of children who do not take hydrocortisone medication.

What is cortisol and how is adrenal insufficiency treated?

Patients with adrenal insufficiency do not make enough cortisol. Cortisol is a hormone (a chemical messenger in the blood) that has many essential functions in our body. It is one of our energy hormones and helps us to fight infection and recover from injuries. Cortisol also helps to control our blood pressure and metabolism (how we store and use fat, protein and carbohydrates for energy), and we know these are very important for our cardiovascular health (the health of our heart and blood vessels).

If your cortisol levels are low, we replace it with the medicine called hydrocortisone. We try to copy the cortisol levels that our body would normally produce but this can

be very difficult. In this study we would like to see if we can utilise salivary sampling to help guide management.

Why has my child been chosen?

Our records show that your child has adrenal insufficiency and they are treated with hydrocortisone. This follows on from the saliva samples obtained as part of the GRACE study.

What will happen if my child and I agree to take part?

We will send out a salivary sampling kit for you to take saliva measurements at home, first thing in the morning, prior to your child's hydrocortisone dose and then a further sample two hours later. These can then be returned to the hospital at your earliest convenience.

What are the benefits to my child taking part in this study?

We hope that these results will help give a greater understanding of the health of children with adrenal insufficiency.

Will my child have any extra appointments or blood tests if they join the study?

We will aim for these samples to be returned at a time when you will be visiting the hospital for clinical reasons.

Are there any risks to my child if they join the study?

The salivary sampling kit is age appropriate. These are safe in paediatric practice.

Does my child have to take part, and can we change our mind?

If you do not wish your child to take part in the study, you do not have to give a reason. Your child will still receive the best available care. If you decide to take part and new information becomes available, your doctor will discuss this with you. If at any point you wish to withdraw you do not have to offer a reason and the assurance of receiving the best possible care available still applies.

What will happen to my child's information?

In this research study we will use information from your child's medical records. We will only use information that we need for the research study. We will let very few people know your child's name or contact details, and only if they really need it for this study. Everyone involved in this study will keep your child's data safe and secure. We will also follow all privacy rules. At the end of the study we will save some of the data in case we need to check it and to support further research. We will make sure no-one can work out who your child is from the reports we write.

How will we (Alder Hey Children's NHS Foundation Trust) use information about your child?

We will need to use information from your child's medical records for this research project. This information will include your child's name, date of birth, Alder Hey medical record number, NHS number and contact details. People will use this information to do the research or to check your child's records to make sure that the research is being done properly. People who do not need to know who your child is will not be able to see your child's name or contact details. Your child's data will have a code number instead. We will keep all information about your child safe and secure. Once we have finished the study, we will keep some of the data so we can check the results. We will write our reports in a way that no-one can work out that your child took part in the study.

What are your choices about how your information is used?

- You and your child can stop being part of the study at any time, without giving a reason, but we will keep information about your child that we already have.
- We need to manage your child's records in specific ways for the research to be reliable. This means that we won't be able to let you or your child see or change the data we hold about them.

- If you and your child agree to take part in this study, your child will have the option to take part in future research using your child's data saved from this study. Your child can stop being part of the study at any time, without giving a reason, but we will keep information about them that we already have.

Where can you find out more about how your information is used?

You can find out more about how we use your information at www.hra.nhs.uk/information-about-patients/ or by asking a member of the research team.

What will happen to my child's blood samples?

The results of these samples will be recorded. The samples will be stored in Alder Hey for 5 years. Only research staff and biochemist staff who are analysing the results will have access to these samples.

What will happen at the end of the research study?

The results of the study will be analysed and shared with doctors across the world who care for children and young people with adrenal insufficiency. The researchers will publish the results in a medical journal and present it at medical conferences. Any results that have an impact on your child will be communicated by the researchers to your child's doctor and to you.

Who is doing this research study?

The study is funded by the Hugh Greenwood charity. The study is being run at Alder Hey Children's Hospital. It has been organised by Alder Hey Children's Hospital, University of Liverpool, and Liverpool Centre for Cardiovascular Science. This research has been approved by a research ethics committee, who has agreed the study is being conducted in an appropriate manner.

What if there is a problem?

If you have any questions or concerns about the study, please contact Dr Julie Park who is leading the study at your child's hospital. If you have any questions concerning your rights in this research study, you may contact the Patient Advisory Liaison Service (PALS) at Alder Hey Children's hospital.

Thank you for reading this. Please ask us any questions that you have about the study.

Contact details: Dr Julie Park, Dept of Endocrinology, Alder Hey Children's NHS Foundation Trust, East Prescot Road, Liverpool L145AB 01512284811 Ext 2335/2281

Julie.park@alderhey.nhs.uk

9.8. Example of consent form used for GRACE 2

Centre Name and Number:	
Patient's study number:	
Patient's initials: _ _ _	Patient's Date of Birth: _ _/_ _ /_ _ _ _

Glucose Regulation and Cardiovascular Health in Adrenal Insufficiency (part 2)

CONSENT FORM FOR RESEARCH

For parent/person with parental responsibility

Version 1.0, 8th February 2022

Name of researcher: **Please initial box**

1. I confirm that I have read and understand the information sheet (version 1.0), dated 8-2-2022 and 'Additional information' leaflet (version 1.0), dated 8-2-2022 for the above study and have had the opportunity to ask questions. ☐

2. I understand that participation is voluntary and that and that I am free to withdraw my child at any time, without giving a reason, and without my care/child's medical care and legal rights being affected. ☐

3. I understand that my child will be asked to obtain two salivary samples, one on waking and one two hours post their hydrocortisone dose and that these samples will be kept for 5 years, for use only in this study ☐

4. I understand that relevant sections of my child's medical notes and data collected during the study may be looked at by individuals from the research team, regulatory authorities, sponsor, or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my child's records for this purpose. ☐

5. I understand that my child's medical data will be collected for this study and may be used to develop new research and that data protection regulations will be observed. ☐

6. I understand that information about my child (including names) will be kept strictly confidential and that no personal information will be used in the study report or other publications. ☐

7. I agree for my child to take part in the above study. ☐

Name of patient _____

| --- | --- | --- |
Name of parent or guardian	Date	Signature
Name of person taking consent	Date	Signature

9.9. Exercise diary for participants to document hydrocortisone dose, exercise and bedtime throughout the study in GRACE 1 and 2

Patient ID: **Medication, any illnesses, and exercise diary** **GRACE 2 (IRAS: 302412)**

Day	Hydrocortisone Time Taken	Hydrocortisone Dose (mg) and formulation (type)	Normal dose/sick day	Exercise- time and activity	Bedtime
1					
2					
3					
4					
5					
6					
7					

- Please indicate the time that you have taken your medication, what dose you have taken, whether it is sick day or your normal dose and any exercise that you have performed. Thank you

9.10. Summary tables for GRACE 2 by diagnosis

Table 9.4: Table summarising participants 201-210 from GRACE 2

Study ID	**201***	**202**	**203****	**204**	**205**	**206**	**207**	**208**	**209***	**210***
i. Baseline Characteristics										
Age (years)	16	10	15	18	2	10	4	19	19	9
Gender	F	F	M	M	M	M	F	F	F	F
Ethnicity	WC	WC	WC	WC	WC	WC	Mi	WC	WC	WC
Congenital or acquired	Acquired	Acquired	Acquired	Acquired	Congenital	Acquired	Congenital	Acquired	Acquired	Congenital
Surgery	Y	Y	Y	Y		Y		Y	Y	
Chemotherapy	Y		Y	Y						
Radiotherapy	Y			Y						
Proton Beam therapy	Y	Y				Y		Y	Y	
Pituitary function										
TSH deficiency	Y	Y	N	Y	Y	Y	Y	Y	Y	Y
GnRH deficiency	Y	?	Y	Y	?	?	?	Y	Y	?
GH deficiency	Y	Y	N	Y	Y	Y	Y	Y	Y	Y
Diabetes insipidus	Y	Y	N	Y	Y	Resolved	N	N	N	N
ii. Relevant History										
Antenatal history	-		Preeclampsia							
Smoking history	-				parent		parent	parent	parent	
CVD in FDR	-		yes							
FH/participant ↑ lipid	-									
Hydrocortisone (mg/m^2/day)	7.1	10$^{\#}$	9.1	9.1	7.1	7.3	10.7	8.3	11.8	7.9
iii. Glucose parameters										
Mean glucose mmol/L	6.1	5.4	6.0	5.9	6.0	5.9	5.3	5.7	5.9	6.2
% glucose<3mmol/L	0.0	0.0	0.0	0.0	0.1	0.0	0.8	0.0	0.3	0.0
% glucose>10mmol/L	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.2	0.0
iv. Cardiovascular variables										
Dyslipidaemia			yes	yes	yes	yes		yes	yes	-
Insulin resistance	yes		yes							-
↑ VWF antigen and activity	yes			yes	-	-	-			-
BMI percentile (>2SDS)	yes	yes	yes	yes	yes	yes		yes		-
Clinic HTN (≥95th centile)			Diastolic						Diastolic	

Ambulatory HTN (≥95th centile)				-	-	-	-			-
Non-dipping on ABPM	yes	yes								
↑ cIMT (≥75th centile)	>95th	75th-90th	50-75th	50th-75th	-	75th	-	50th	50th	90th-95th
↓ FMD (<7.7%)					-		-			
v. Salivary outcomes										
Mean cortisol nmol/L	2.8	7.6	5.4	17.0	17.0	25.2	-	7.5	8.3	24.6
Mean cortisone nmol/L	9.8	14.1	20.2	16.8	20.1	16.8	17.4	41.9	23.8	10.4

Abbreviations – ID: identifier; WB: White British; Mi: mixed race; BA: Black African; M: male; F: female; TSH: thyroid stimulating hormone; GnRH: gonadotrophin releasing hormone; PP: precocious puberty; GH: growth hormone; u: unknown; CVD: cardiovascular disease; FDR: first-degree relative; FHx: family history; mg/m^2/day: milligrams per meter squared per day; mcg/m^2/day: micrograms per metre squared per day; mmol/L: millimoles per litre; VWF: won Willebrand antigen and activity; BMI SDS: body mass index standard deviation score; CGM: continuous glucose monitoring system; HTN: hypertension; CIMT: carotid intima-media thickness; FMD: flow-mediated dilatation; nmol/L: nanomoles per litre.

*on metformin **on anti-hypertensives $^{\#}$On Plenadren

Table 9.5: Table summarising participants 211-220 from GRACE 2

Study ID	**211**	**212**	**213**	**214**	**215**	**216**	**217**	**218****	**219**	**220**
i. Baseline Characteristics										
Age (years)	13	8	14	9	18	11	13	15	11	14
Gender	M	M	F	M	M	F	F	M	F	F
Ethnicity	WC	WC	WC	WC	WC	WC	WC	WC	WC	BA
Congenital or acquired	Congenital	Acquired	Acquired	Acquired	Acquired	Congenital	Congenital	Acquired	Acquired	Acquired
Surgery		Y	Y	Y	Y			Y	Y	Y
Chemotherapy					Y			Y		
Radiotherapy								Y		
Proton Beam therapy			Y	Y	Y				Y	
Pituitary function										
TSH deficiency	Y	Y	Y	Y	Y	N	Y	Y	Y	Y
GnRH deficiency	?	?	Y	?	PP	N	Y	N	?	Y
GH deficiency	Y	Y	Y	N	Y	Y	Y	Y	Y	Y
Diabetes insipidus	N	Y	Y	Y	Y	Y	N	N	Y	Y
ii. Relevant History										
Antenatal history					u		u			
Smoking history		parent				parent	parent			
CVD in FDR										u
FH/participant ↑ lipid										
Hydrocortisone (mg/m^2/day)	8.0	7.5	7.4	7.7	8.8	6.8	10.9	9.1	8.5	9.2
iii. Glucose parameters										
Mean glucose mmol/L	6.0	5.6	5.8	4.9	6.9	6.3	5.7	6.5	6.2	
% glucose<3mmol/L	0.0	0.0	0.2	1.4	0.0	0.0	0.0	0.0	0.0	0.5
% glucose>10mmol/L	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.4	0.0	0.0
iv. Cardiovascular variables										
Proatherogenic Lipid profile	yes		yes	yes	yes			yes	yes	yes
Insulin resistance			yes	yes	yes		yes			
↑ VWF antigen and activity	yes								yes	
BMI percentile (>2SDS)		yes	yes	yes		yes				
Clinic HTN (≥95th centile)			Systolic							
Ambulatory HTN (≥95th centile)						-				

Non-dipping on ABPM	yes	yes	yes	yes					yes	yes
↑ cIMT (≥75th centile)	90th	75th-90th	75th	75th	>95th	75th	>95th	>95th	75th -90th	75th
↓ FMD (<7.7%)		Yes		yes	yes	-		-	-	
v. Salivary outcomes										
Mean cortisol nmol/L	27.0	17.9	16.9	18.4	13.5	26.8	7.2	6.8	6.3	8.9
Mean cortisone nmol/L	15.9	11.6	19.3	9.9	20.4	10.8	13.7	18.2	11.2	19.9

Abbreviations – ID: identifier; WB: White British; Mi: mixed race; BA: Black African; M: male; F: female; TSH: thyroid stimulating hormone; GnRH: gonadotrophin releasing hormone PP: precocious puberty; GH: growth hormone; u: unknown; CVD: cardiovascular disease; FDR: first-degree relative; FHx: family history; mg/m^2/day: milligrams per meter squared per day; mcg/m^2/day: micrograms per metre squared per day; mmol/L: millimoles per litre; VWF: won Willebrand antigen and activity; BMI SDS: body mass index standard deviation score; CGM: continuous glucose monitoring system; HTN: hypertension; CIMT: carotid intima-media thickness; FMD: flow-mediated dilatation; nmol/L: nanomoles per litre.

*on metformin **on anti-hypertensives #On Plenadren

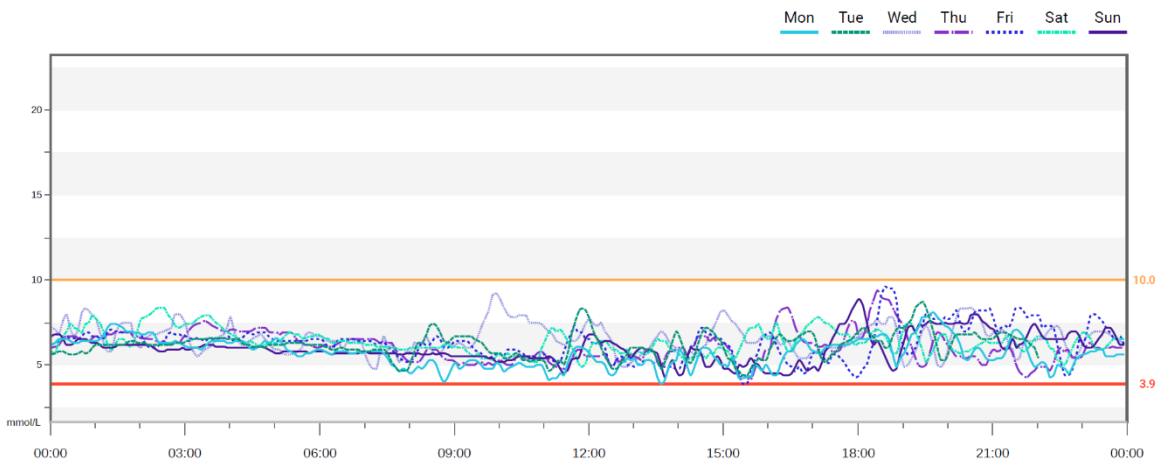
9.11. GRACE 2: Hydrocortisone doses, AGPs and salivary cortisol and cortisone profiles

Participant 201

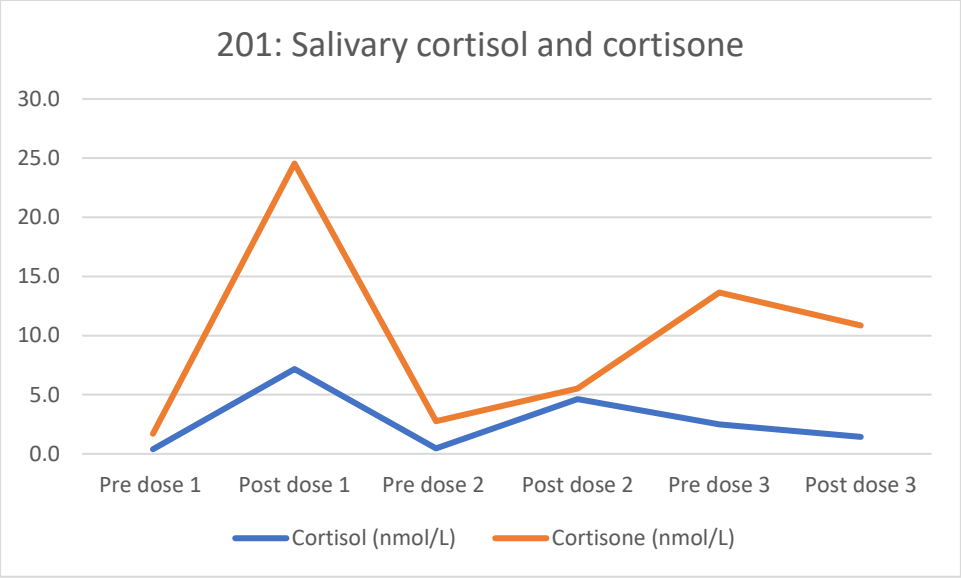
Hydrocortisone formulation: Standard

Doses: 5mg at 7am, 5mg, 1.30pm, 5mg at 6pm (7.1mg/m^2/day).

AGP:



Salivary profile:

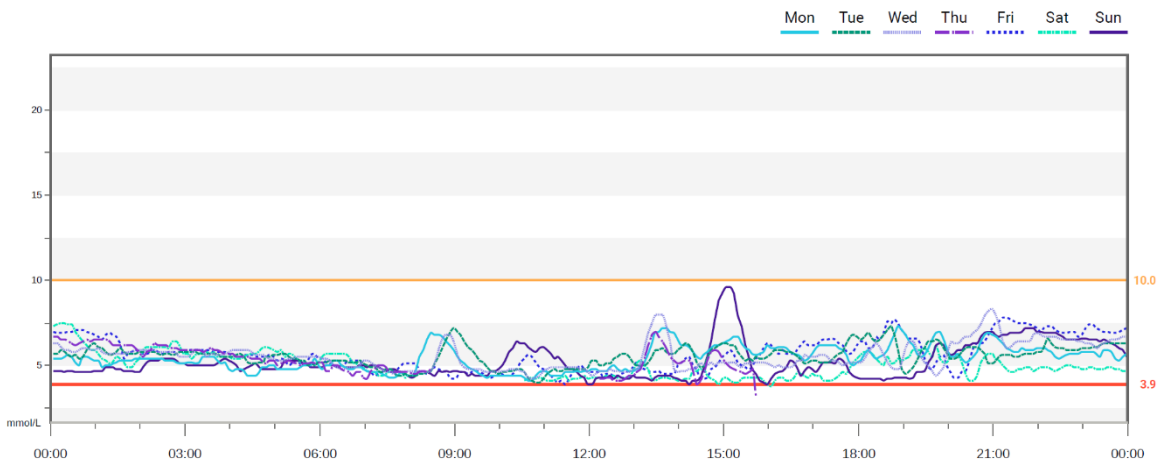


Participant 202:

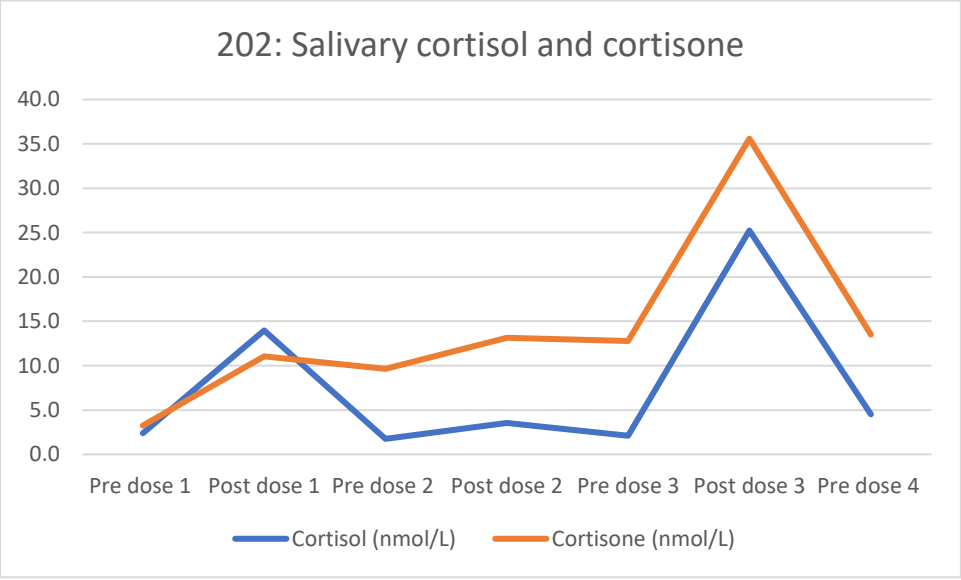
Hydrocortisone formulation: All Plenadren®

Doses: 5mg at 7am, 5mg at 12pm, 5mg at 6pm (10mg/m^2/day)

AGP:



Salivary profile:

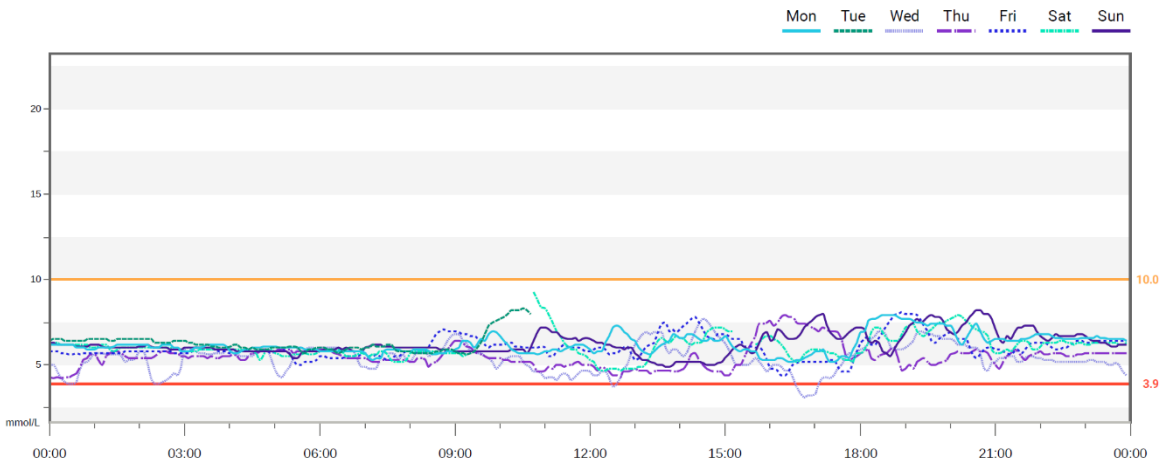


Participant 203:

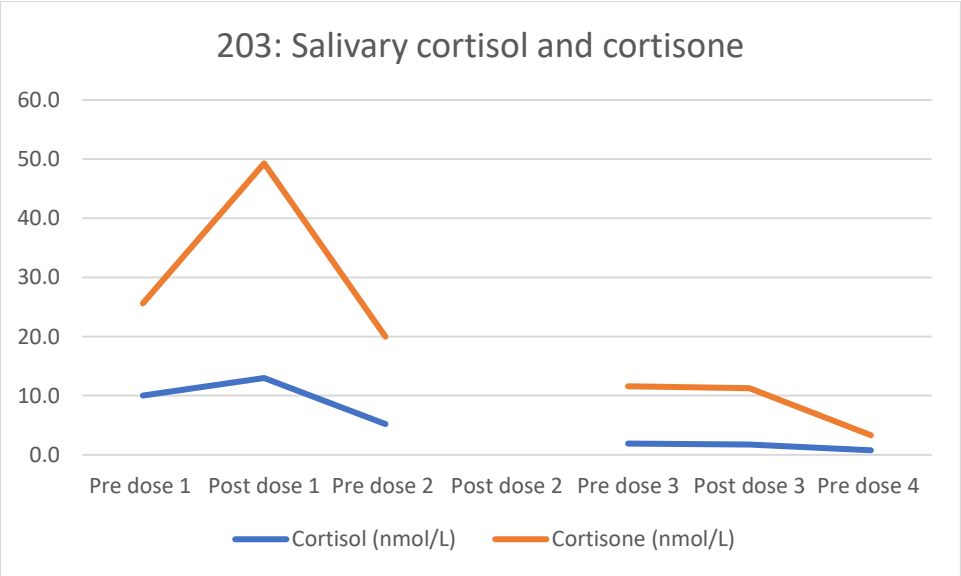
Hydrocortisone formulation: Standard

Doses: 10mg at 9am, 5mg at 3pm, 5mg at 8pm (9.1mg/m^2/day)

AGP:



Salivary profile:

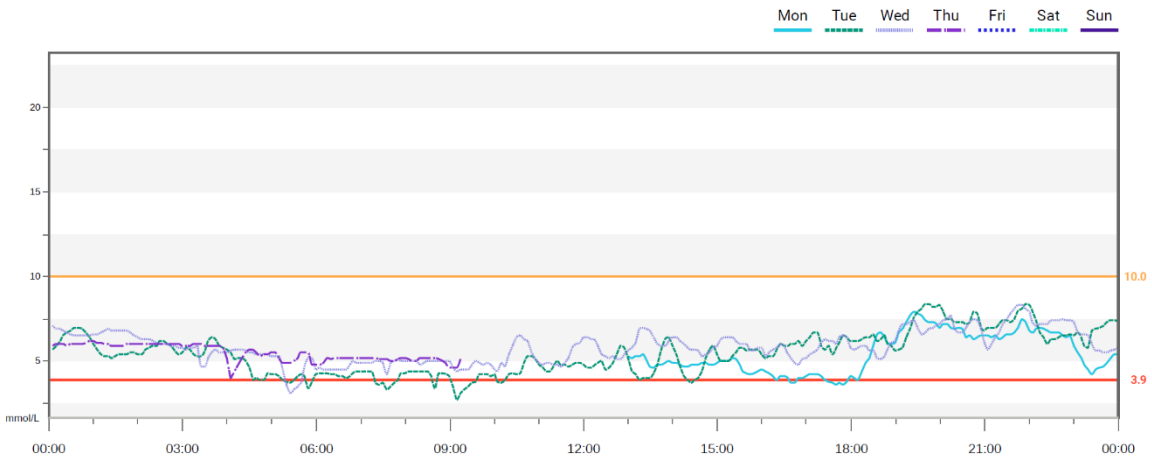


Participant 204:

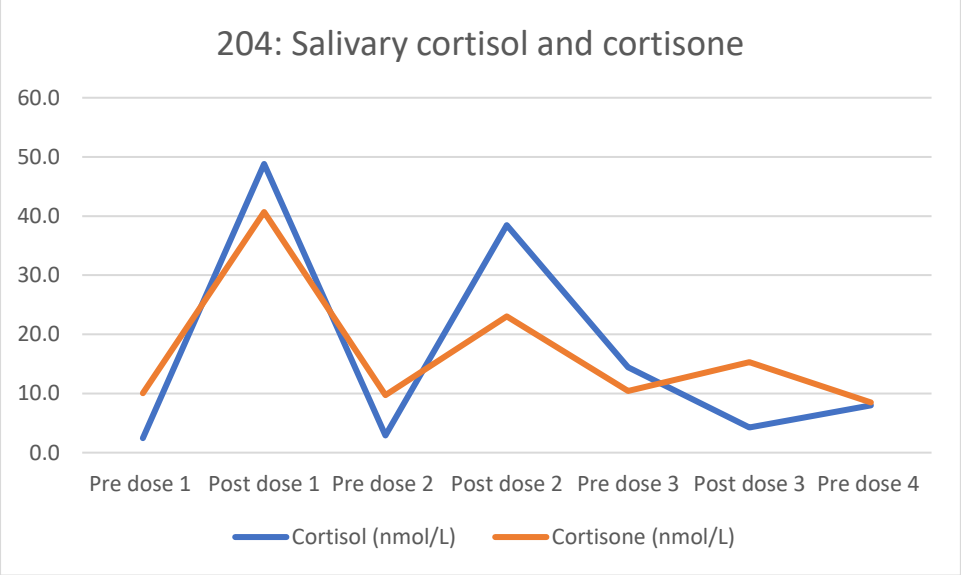
Hydrocortisone formulation: Standard

Doses: 10mg at 9am, 5mg at 1pm, 5mg at 8pm (9.1mg/m^2/day)

AGP:



Salivary profile:

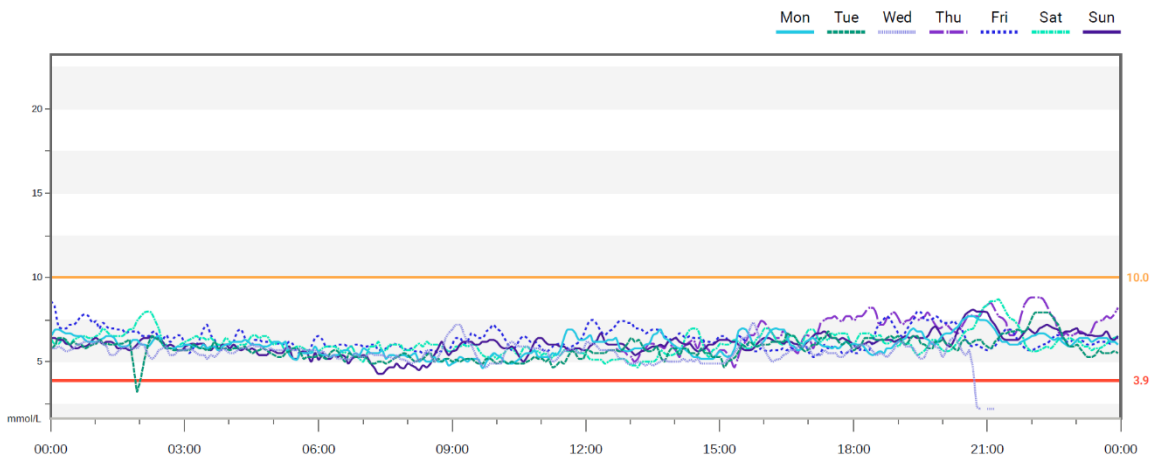


Participant 205:

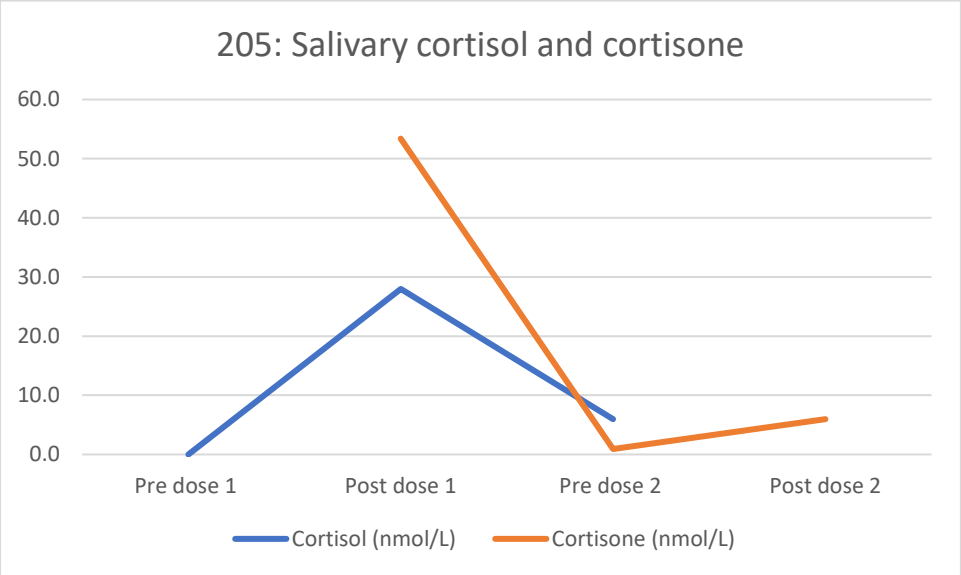
Hydrocortisone formulation: Standard

Doses: 1.5mg at 8am, 1.5mg at 3pm, 1.5mg at 8pm (7.1mg/m^2/day)

AGP:



Salivary profile:

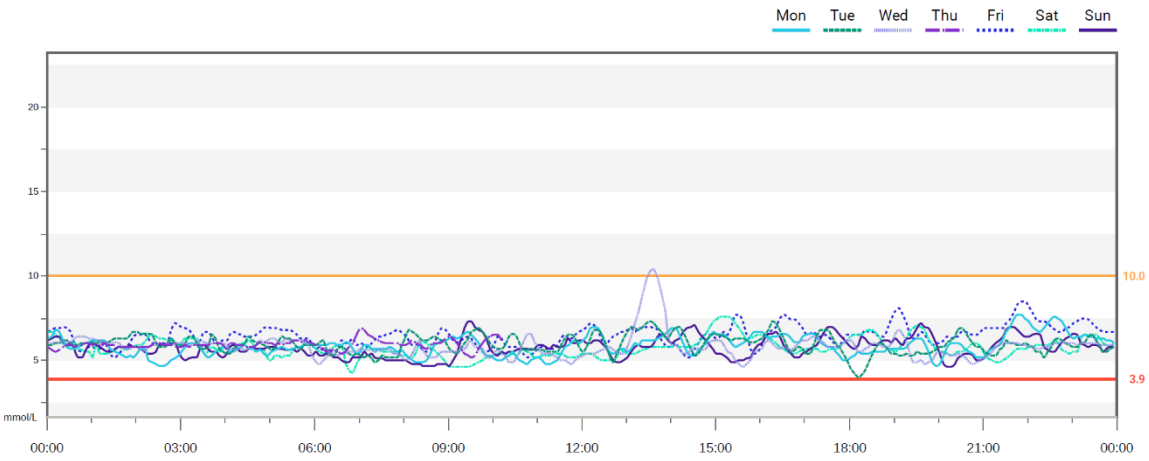


Participant 206:

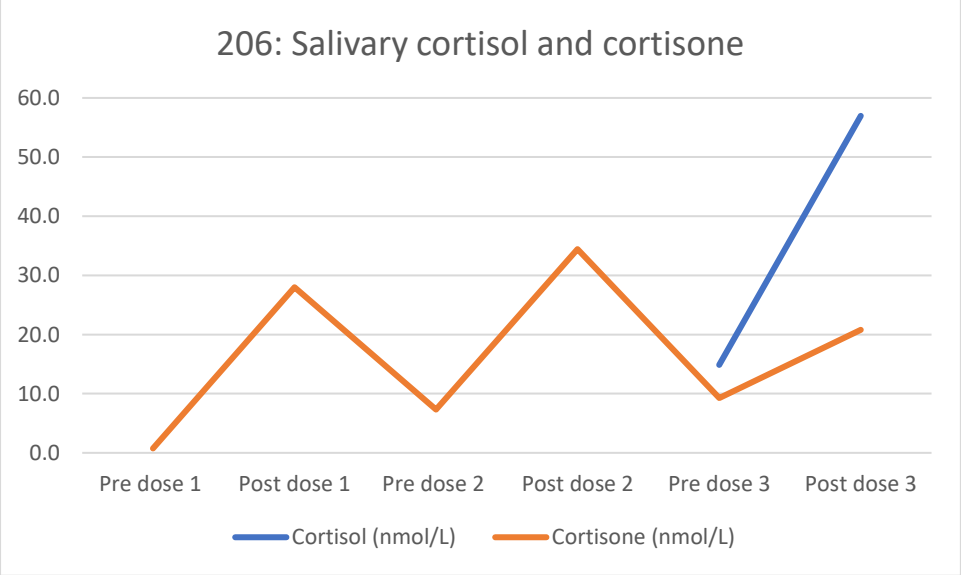
Hydrocortisone formulation: Standard

Doses: 3.5mg at 7.30am, 3.5mg at 3pm, 2.5mg at 8pm (7.3mg/m^2/day)

AGP:



Salivary profile:

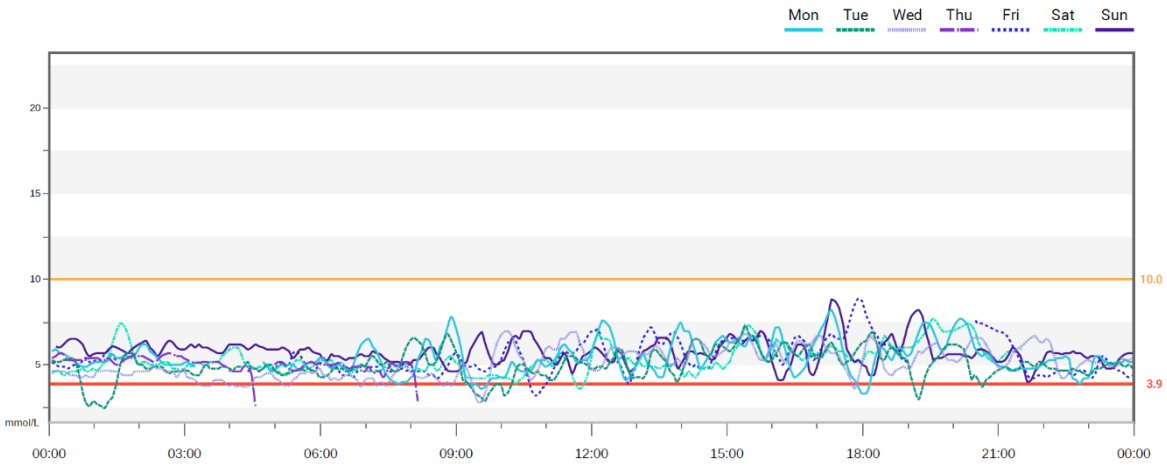


Participant 207:

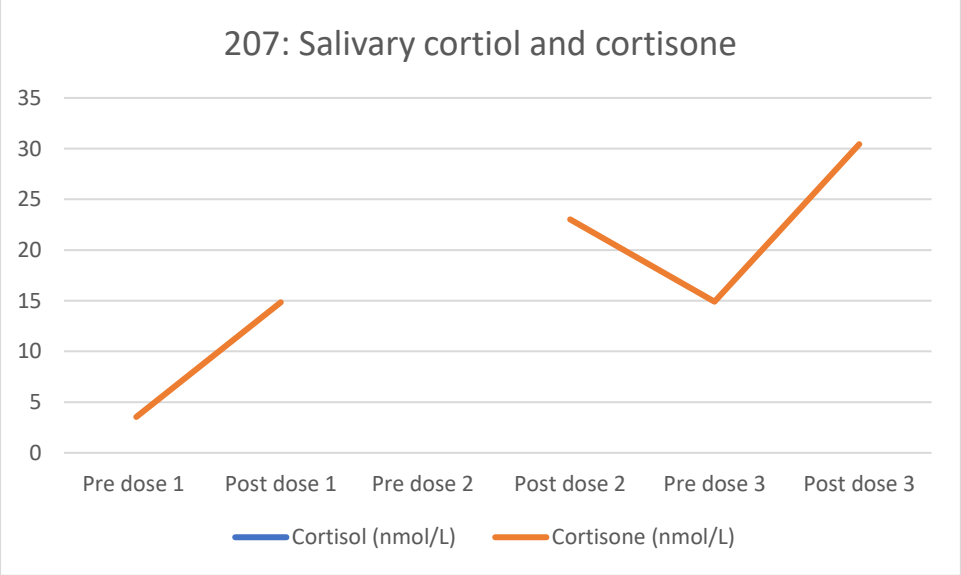
Hydrocortisone formulation: Standard

Doses: 3mg at 7am, 2.5mg at 12.30pm, 1mg at 6pm (10.7mg/m^2/day)

AGP:



Salivary profile:

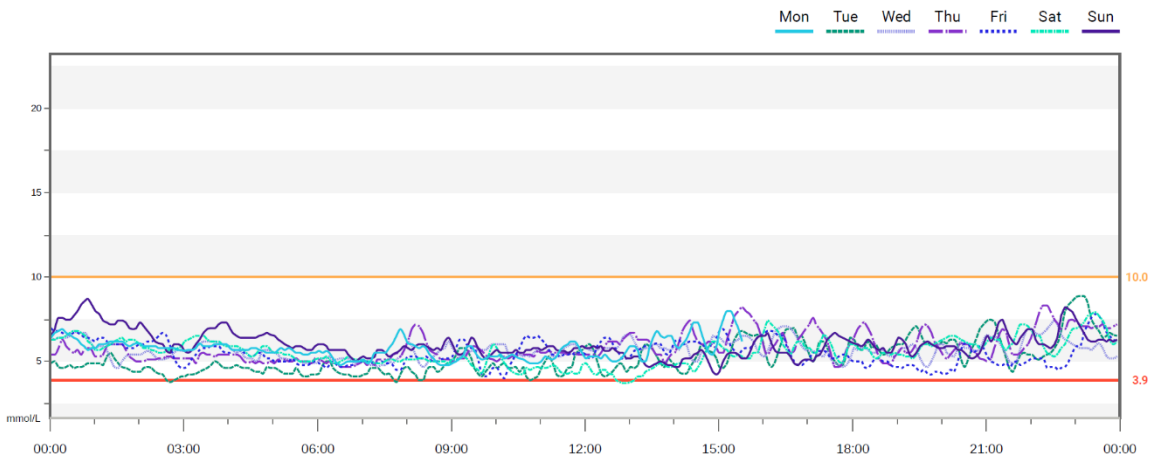


Participant 208:

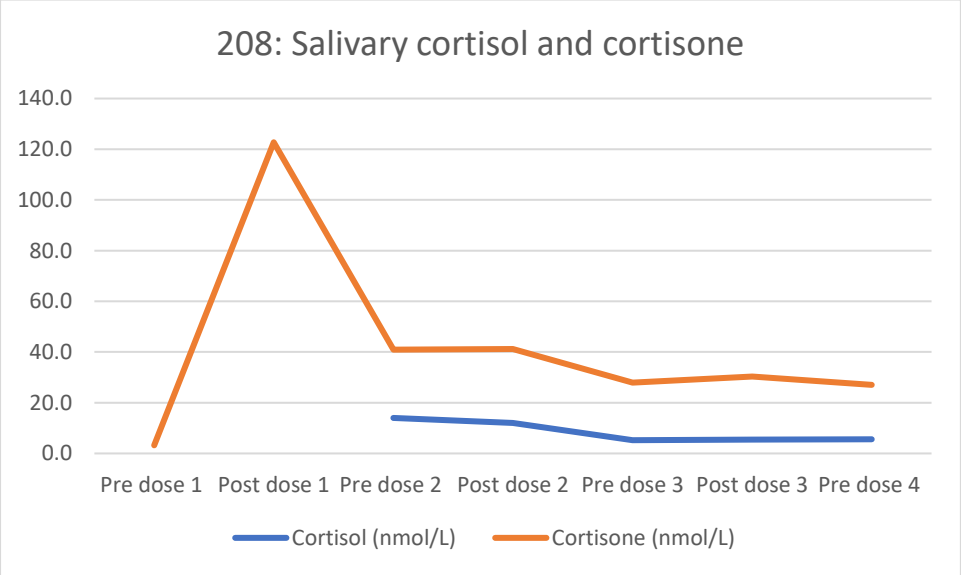
Hydrocortisone formulation: Standard

Doses: 10mg at 7am, 5mg at 1pm, 2.5mg at 6pm (8.3mg/m^2/day)

AGP:



Salivary profile:

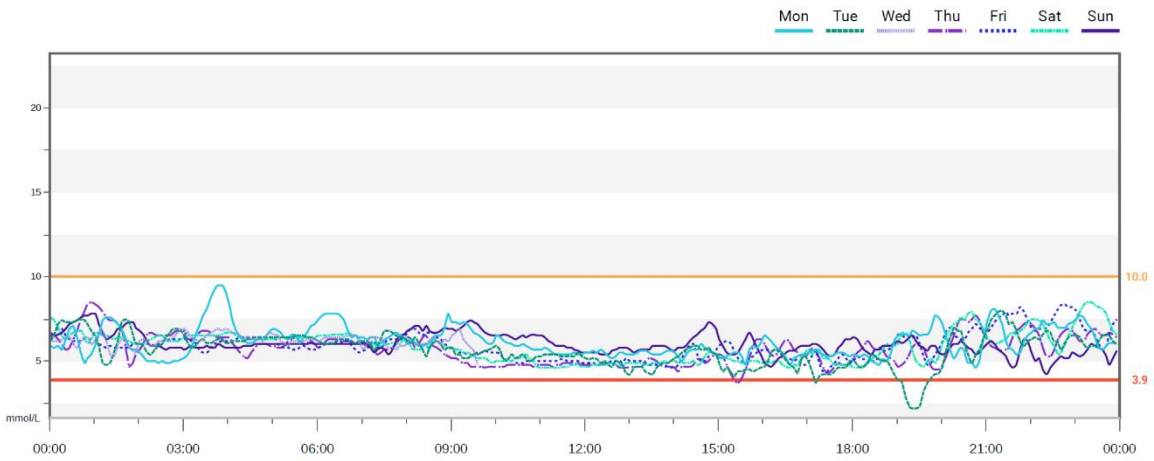


Participant 209:

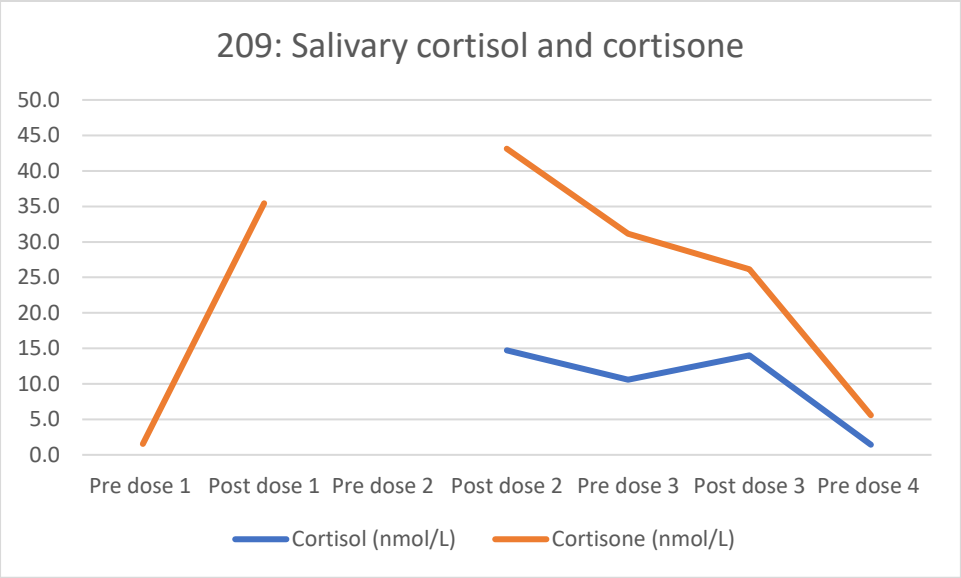
Hydrocortisone formulation: Standard

Doses: 7.5mg 6am, 5mg at 11am, 2.5mg at 3pm, 5mg at midnight (11.8mg/m^2/day)

AGP:



Salivary profile:

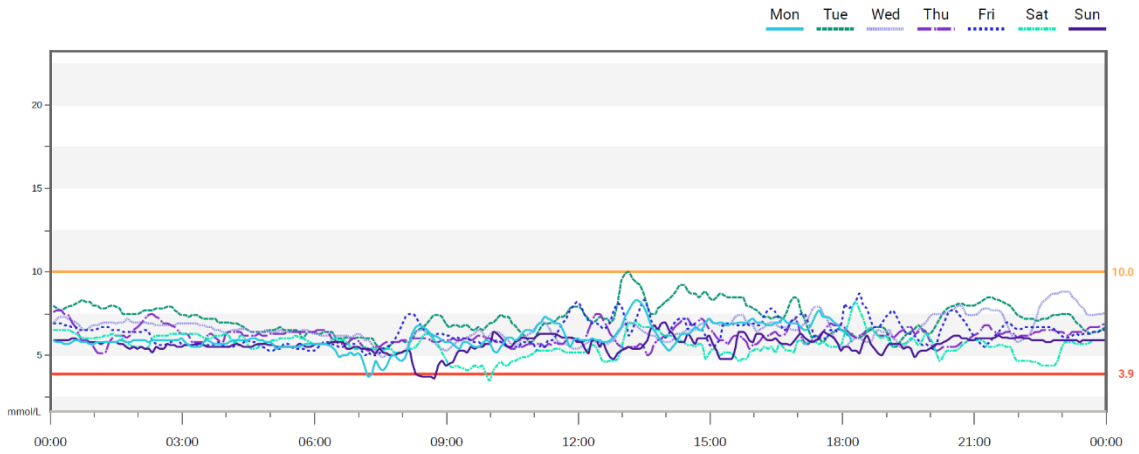


Participant 210:

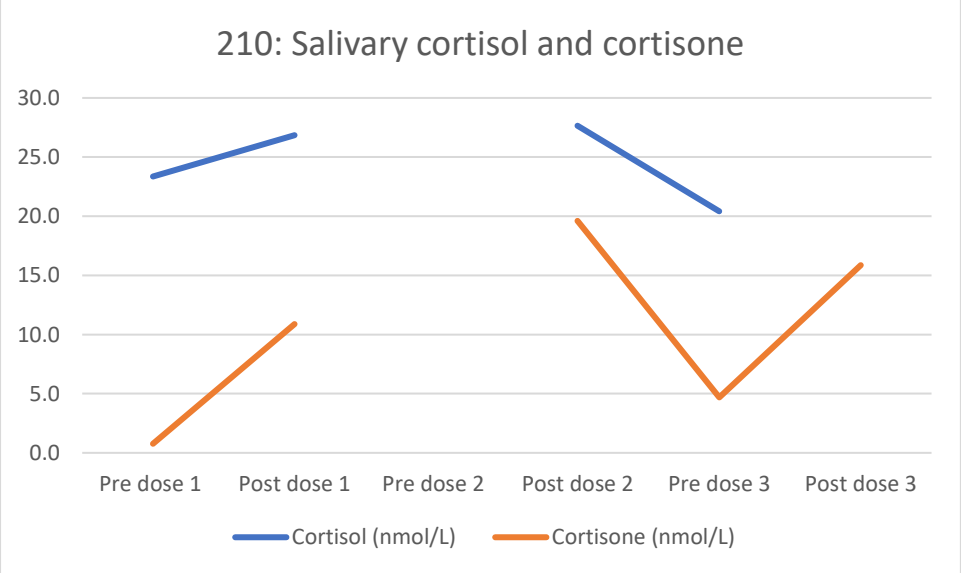
Hydrocortisone formulation: Standard

Doses: 4mg at 7am, 4mg at 12pm, 3mg at 7pm (7.9mg/m^2/day)

AGP:



Salivary profile:

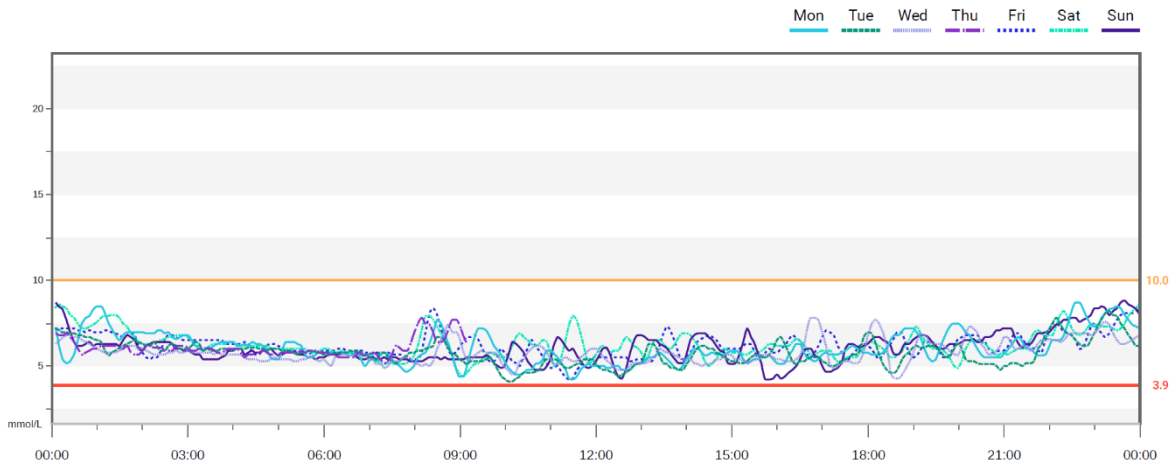


Participant 211:

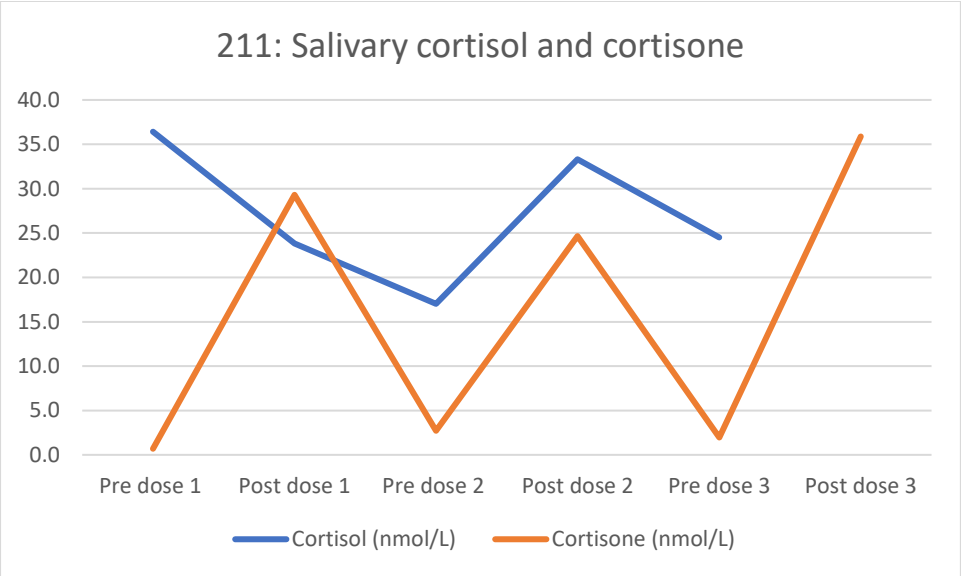
Hydrocortisone formulation: Standard

Doses: 4mg at 7am, 4mg at 12.30pm, 4mg at 8pm (8.0mg/m^2/day)

AGP:



Salivary profile:

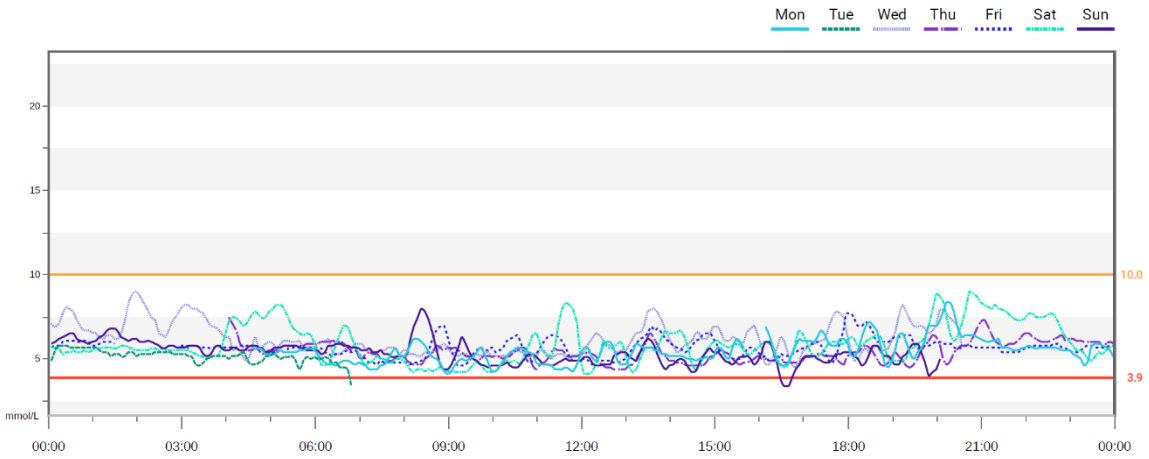


Participant 212:

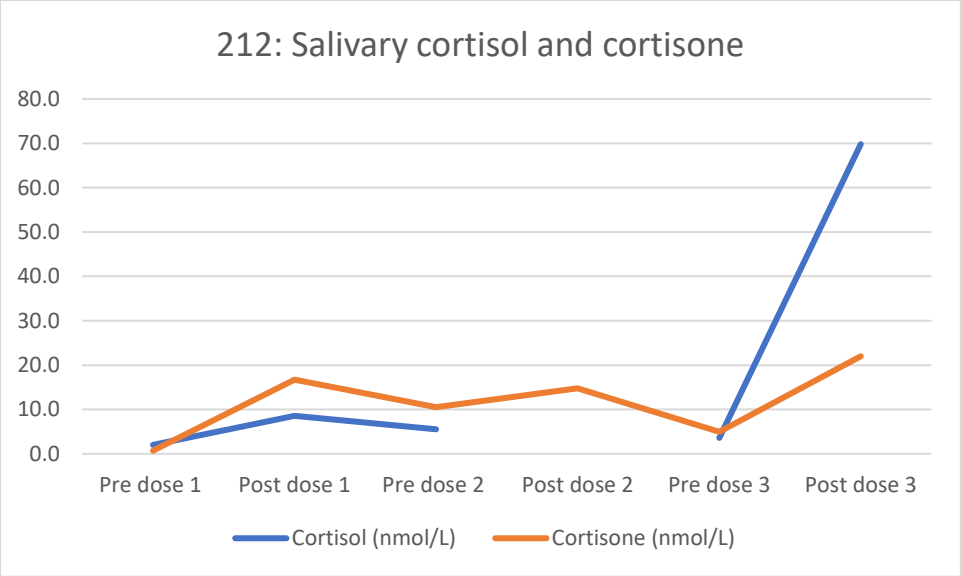
Hydrocortisone formulation: Standard

Doses: 4mg at 7am, 3mg at 12pm, 2mg at 5pm (7.5mg/m^2/day)

AGP:



Salivary profile:

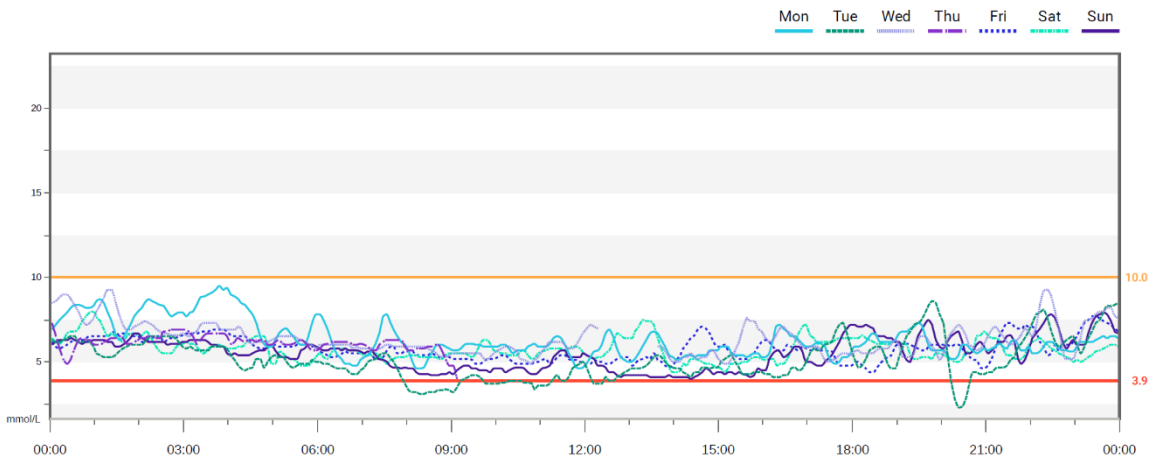


Participant 213:

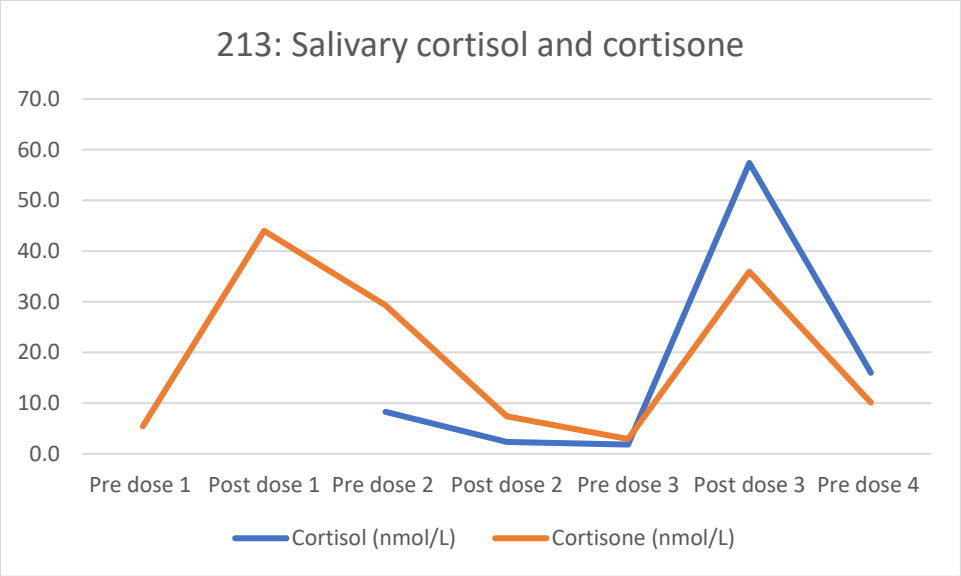
Hydrocortisone formulation: Standard

Doses: 7.5mg at 8.30am, 5mg at 4.30pm, 3mg at 9pm (7.4mg/m^2/day)

AGP:



Salivary profile:

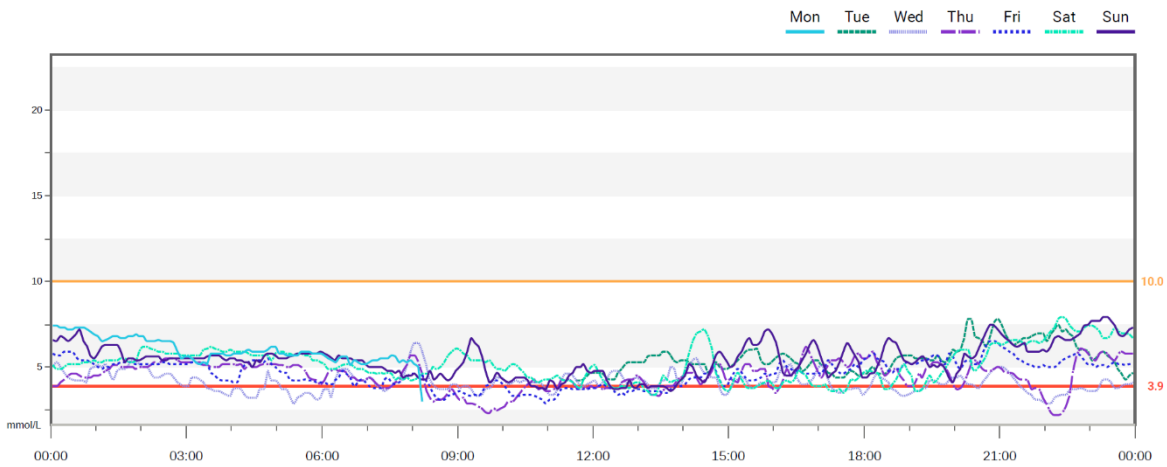


Participant 214:

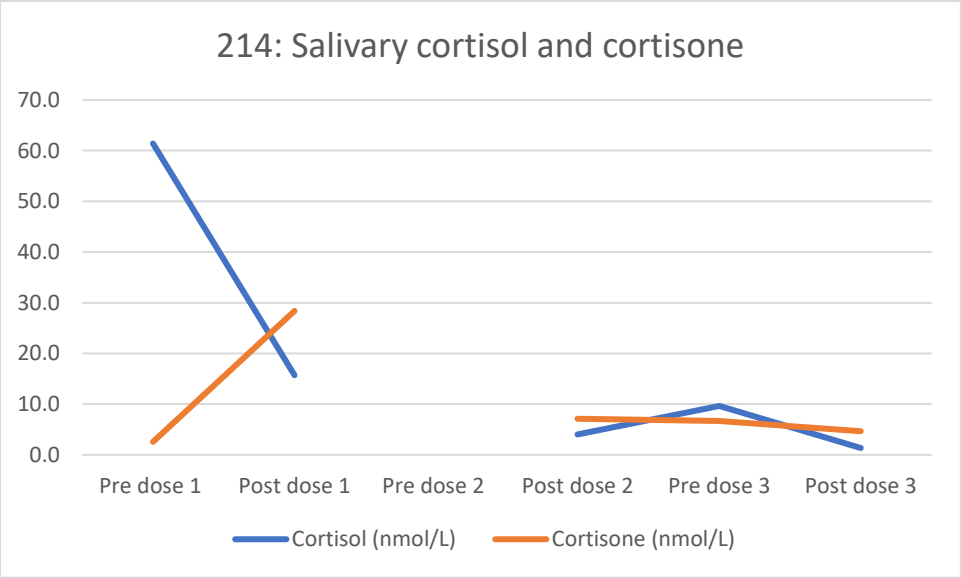
Hydrocortisone formulation: Standard

Doses: 5mg at 8.30am, 2.5mg at 1pm, 2.5mg at 7pm (7.7mg/m^2/day)

AGP:



Salivary profile:

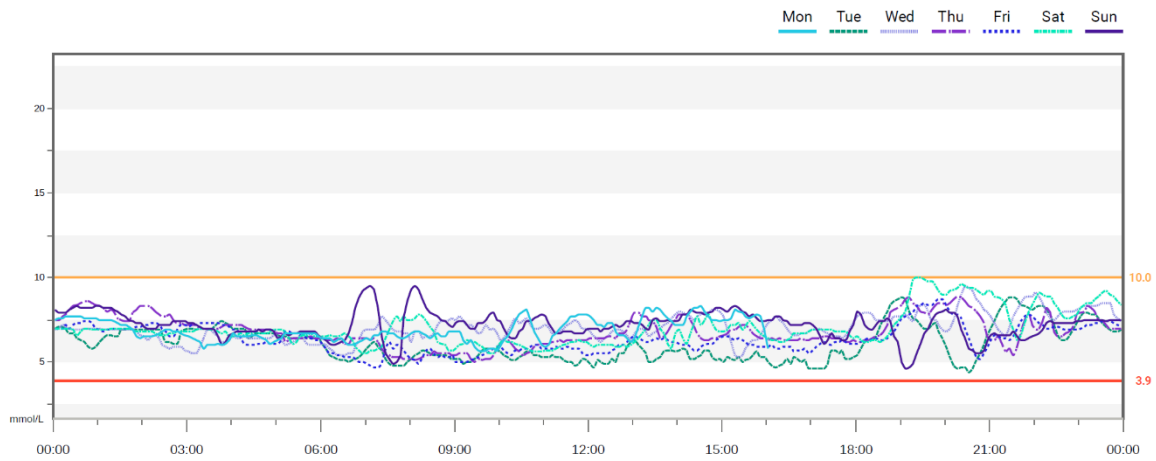


Participant 215:

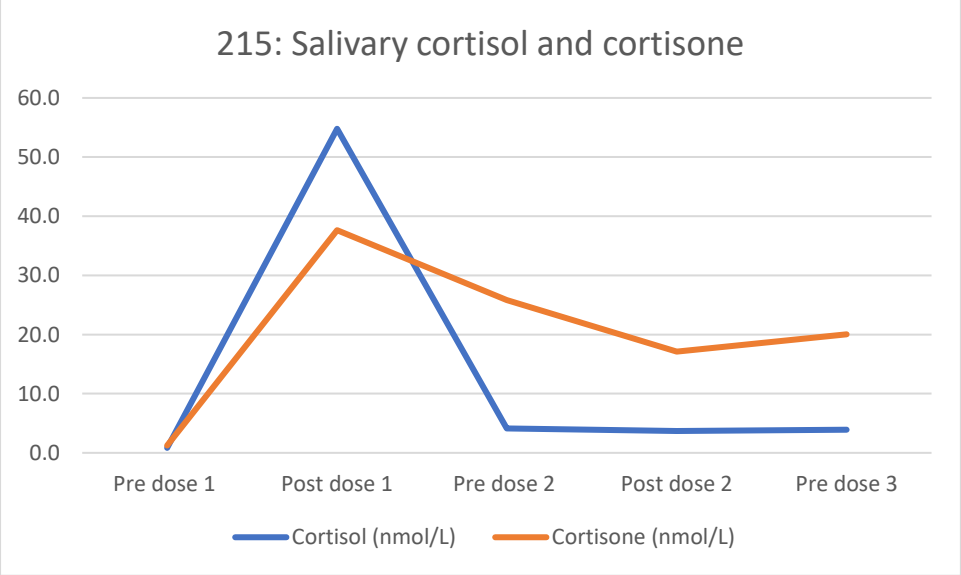
Hydrocortisone formulation: Standard

Doses: 7.5mg at 6.30am, 5mg at 12pm, 5mg at 5pm (8.8mg/m^2/day)

AGP:



Salivary profile:

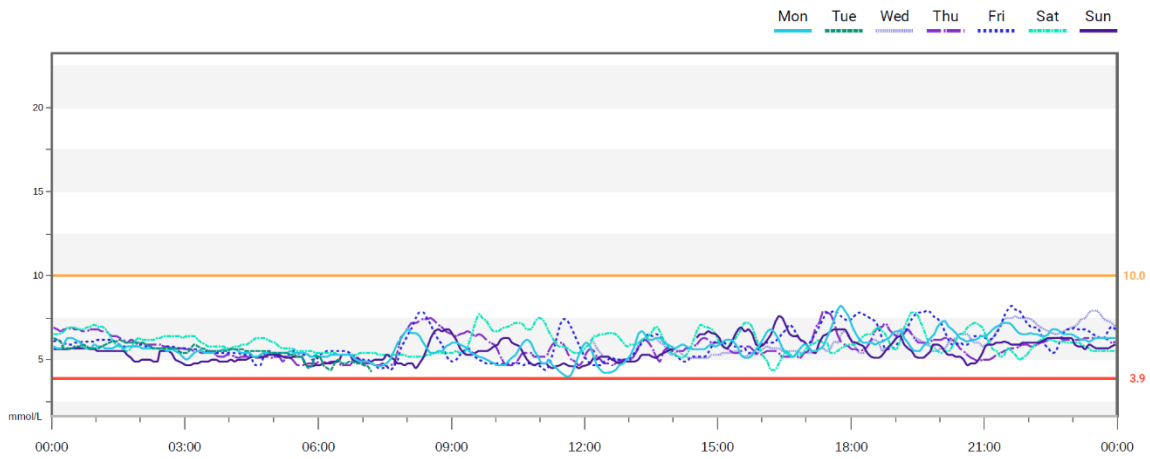


Participant 216:

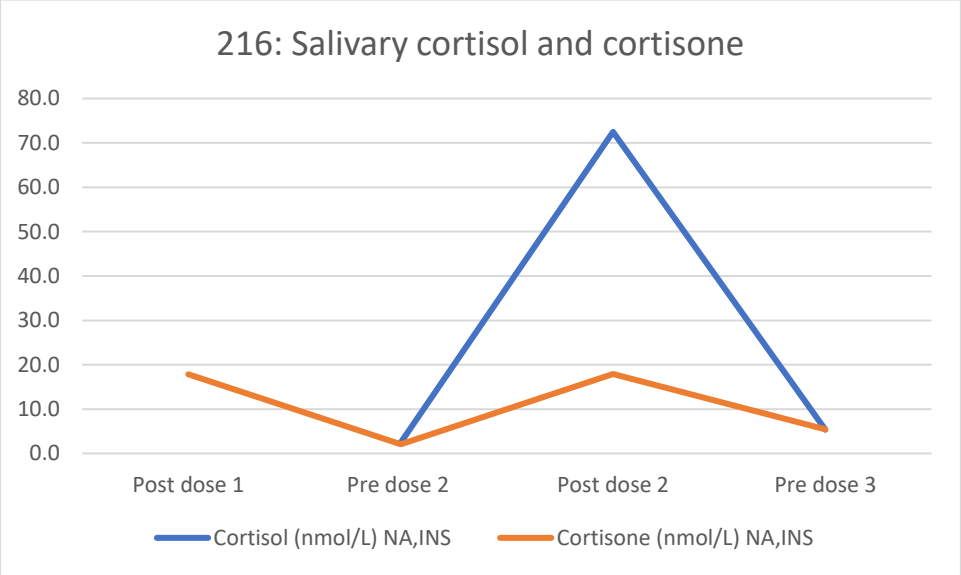
Hydrocortisone formulation: Standard

Doses: 5mg at 8am, 5mg at 2pm, 5mg at 9pm (6.8mg/m^2/day)

AGP:



Salivary profile:

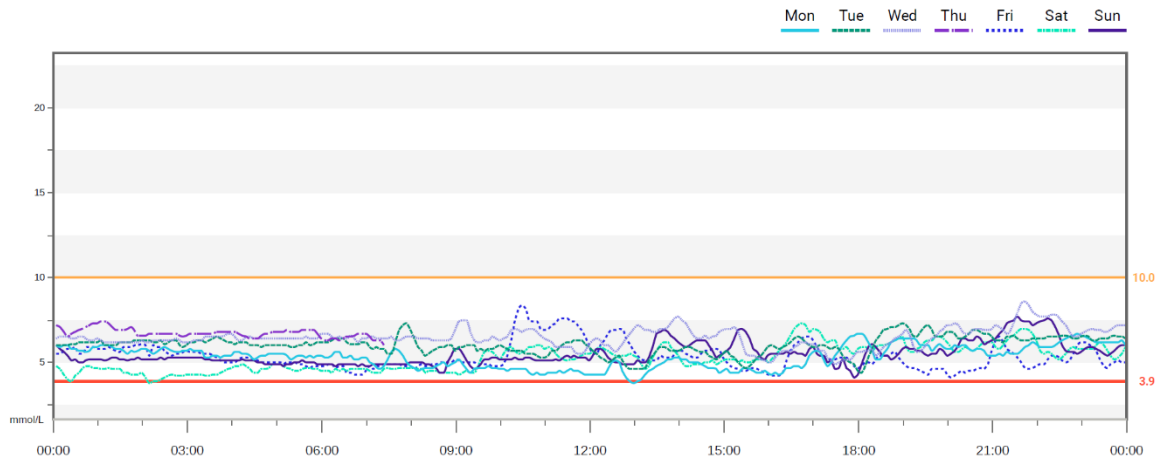


Participant 217:

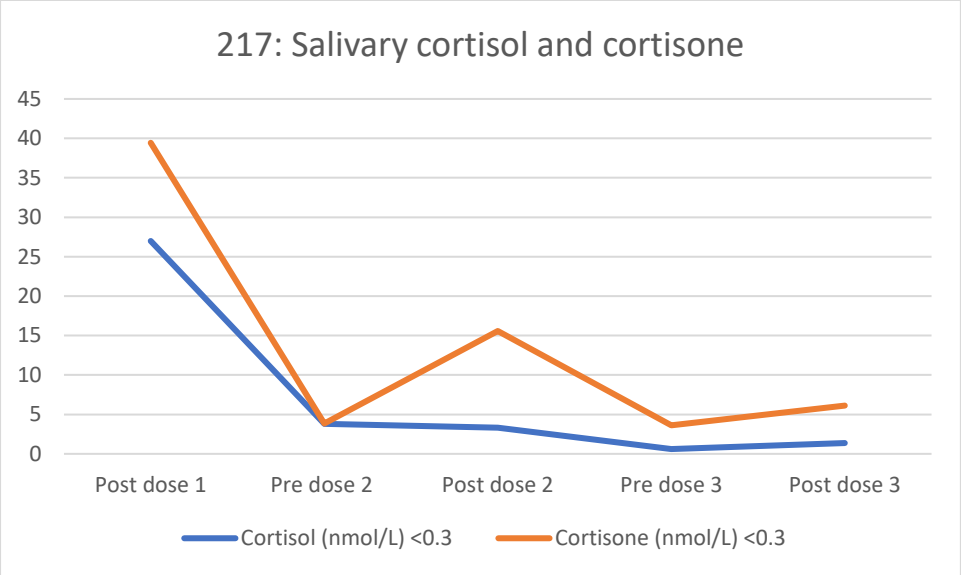
Hydrocortisone formulation: Standard

Doses: 10mg at 7am, 5mg at 12pm, 2.5mg at 6pm (10.9mg/m^2/day)

AGP:



Salivary profile:

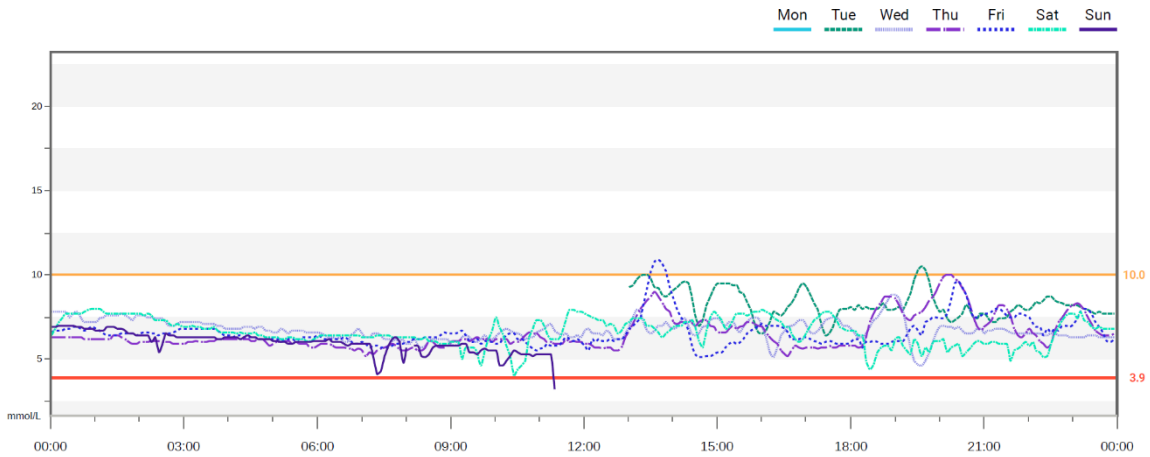


Participant 218:

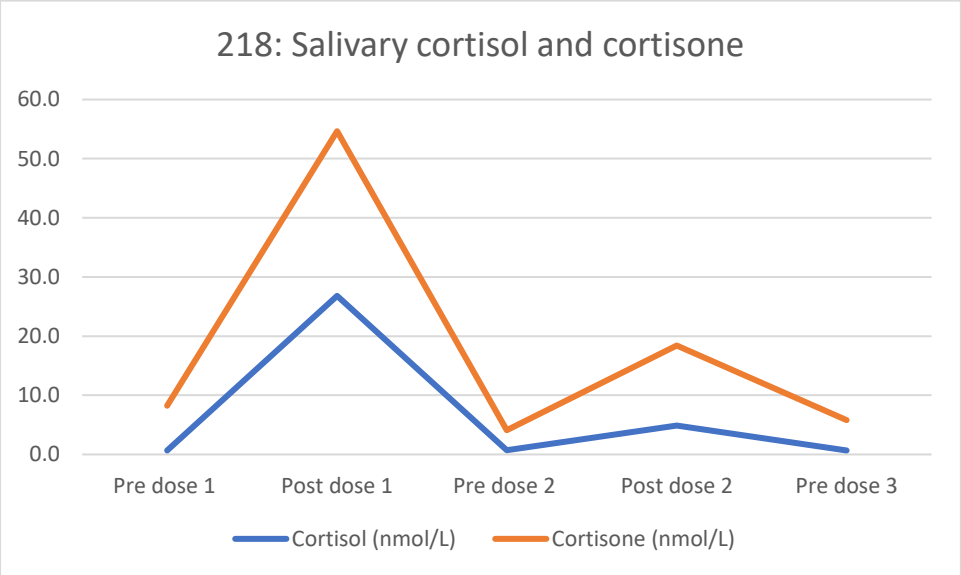
Hydrocortisone formulation: Standard

Doses: 10mg at 7am, 10mg at 4pm (9.1mg/m^2/day)

AGP:



Salivary profile:

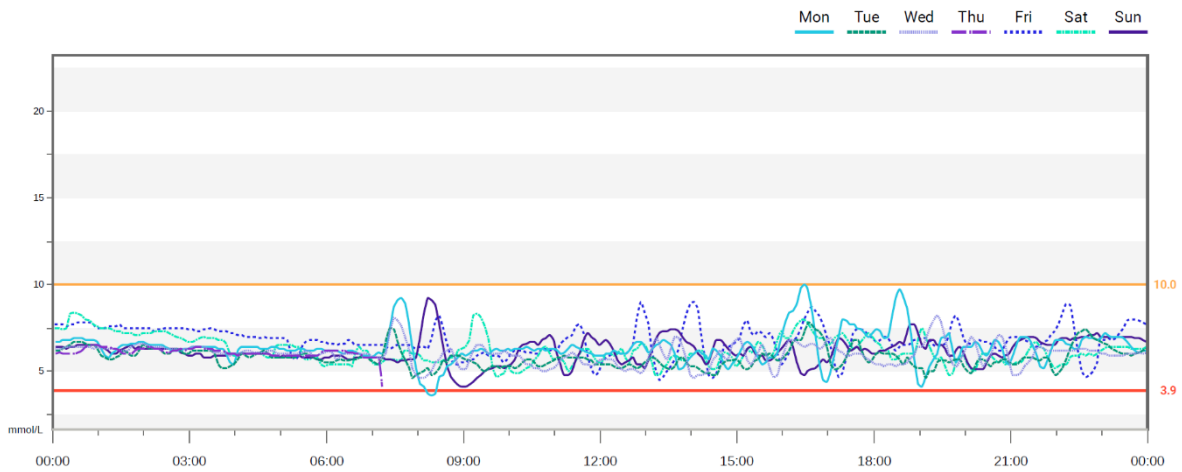


Participant 219:

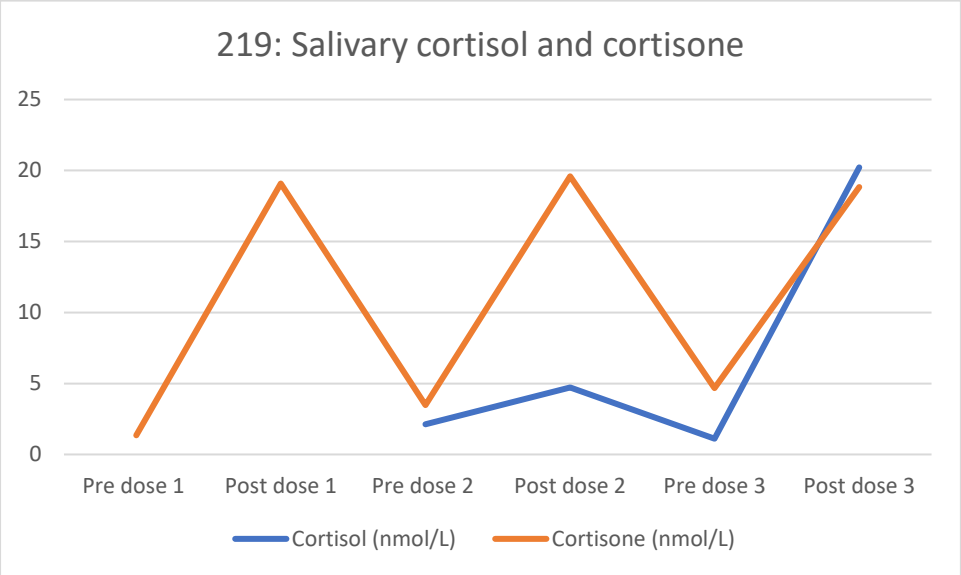
Hydrocortisone formulation: Standard

Doses: 5mg at 8am, 3mg at 2pm, 3mg at 8pm (8.5mg/m^2/day)

AGP:



Salivary profile:

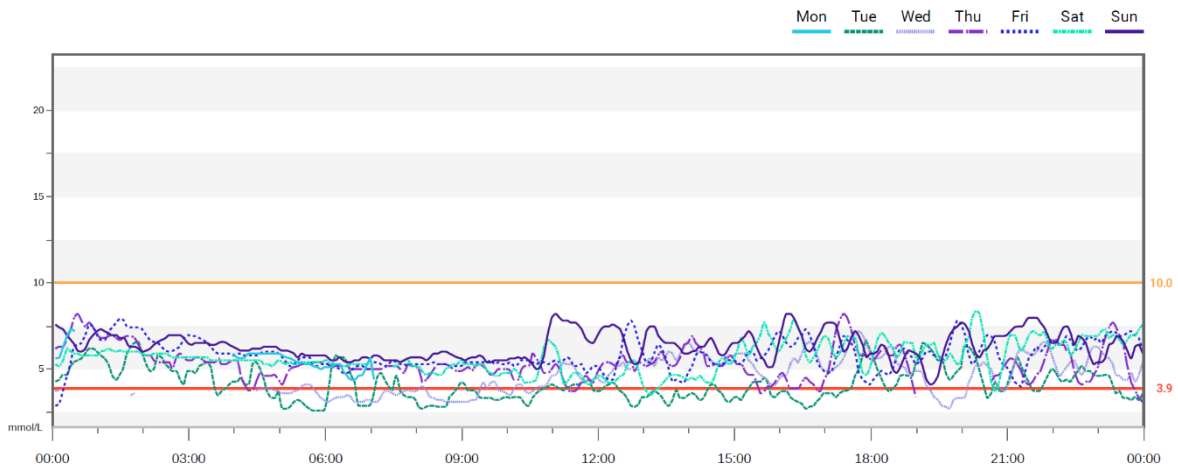


Participant 220:

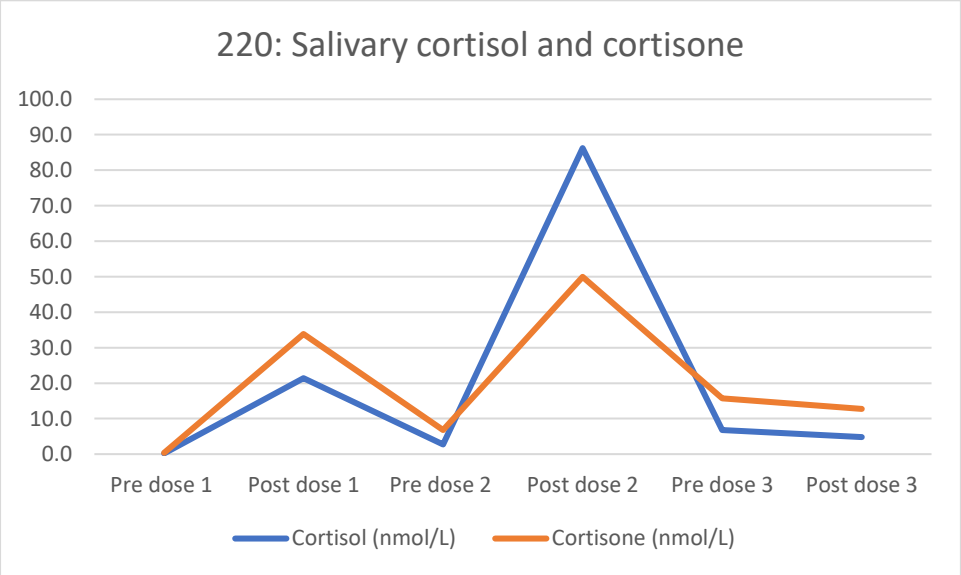
Hydrocortisone formulation: Standard

Doses: 4mg at 8am, 4mg at 1.30pm, 3mg at 5pm (9.2mg/m^2/day)

AGP:



Salivary profile:



9.12. Full peer-reviewed publication for LDSST testing (see chapter 6).

Journal of the Endocrine Society, 2022, 6, 1–7
https://doi.org/10.1210/jendso/bvac043
Advance access publication 19 March 2022
Research Article

Baseline and Peak Cortisol Response to the Low-Dose Short Synacthen Test Relates to Indication for Testing, Age, and Sex

Julie Park,[1,2] Andrew Titman,[3] Gillian Lancaster,[4] Bhavana Selvarajah,[5] Catherine Collingwood,[6] Darren Powell,[6] Urmi Das,[1] Poonam Dhamaraj,[1] Mohammed Didi,[1] Senthil Senniappan,[1] and Joanne Blair[1]

[1]Department of Endocrinology, Alder Hey Children's NHS Foundation Trust, Liverpool, L145AB, UK
[2]Institute of Life Course and Medical Sciences, University of Liverpool, Liverpool, L7 8TX, UK
[3]Department of Mathematics and Statistics, Lancaster University, Lancaster, LA1 4YF, UK
[4]School of Primary, Social and Community Care and Keele Clinical Trials Unit, Keele University, Staffordshire, ST5 5BG, UK
[5]Medical School, University of Liverpool, Liverpool, L69 3GE, UK
[6]Department of Biochemistry, Alder Hey Children's NHS Foundation Trust, Liverpool, L14 5AB, UK

Correspondence: Joanne Blair, MD, Department of Endocrinology, Alder Hey Children's NHS Foundation Trust, E Prescot Rd, Liverpool, L145AB, UK. Email: Jo.blair@alderhey.nhs.uk.

Abstract

Context: Meta-analyses report that the low dose short Synacthen test (LDSST) is more sensitive but less specific than the standard dose test for the diagnosis of adrenal insufficiency, and there are concerns regarding the accuracy of dosing in the LDSST.

Objective: Perform a retrospective, observational study to review the outcomes of LDSSTs performed in a tertiary endocrine service from 2008 to 2014 (N = 335) and 2016 to 2020 (N = 160), and examine for relationships between cortisol measurements and indication for testing, age and sex.

Methods: LDSST were performed by endocrine nurses. Synacthen 500 ng/1.73m2 administered as IV bolus, sampling at 0, 15, 25, and 35 minutes.

Results: Mean (± 1SD) baseline cortisol was 221 ± 120 nmol/L, peak 510 ± 166 nmol/L and increment 210 ± 116 nmol/L. 336 (70%) patients had a normal response (baseline cortisol >100 nmol/L, peak >450 nmol/L.), 78 (16%) a suboptimal response (peak cortisol 350-450 nmol/L) and were prescribed hydrocortisone to during periods of stress only, 67 (14%) an abnormal response (baseline <100nmol/L or peak <350nmol/L) and were prescribed daily hydrocortisone. Basal, peak, and incremental increases in cortisol were higher in females ($P = .03$, $P < .001$, $P = .03$, respectively). Abnormal results occurred most frequently in patients treated previously with pharmacological doses of glucocorticoids or structural brain abnormalities ($P < .001$).

Conclusion: The low prevalence and strong association of abnormal results with indication for testing, suggests that over diagnosis occurred infrequently in this clinical setting.

Key Words: adrenal, Synacthen, hypothalamic-pituitary-adrenal axis testing, HPA, low-dose short Synacthen test, hydrocortisone

Abbreviations: ACTH, adrenocorticotropin; AI, adrenal insufficiency; CYP, children and young people; GH, growth hormone; GHD, growth hormone deficiency; HPA, hypothalamic-pituitary-adrenal; ICS, inhaled corticosteroids; LDSST, low-dose short Synacthen test; SDSST, standard-dose short Synacthen test.

The diagnosis of adrenal insufficiency (AI) in childhood is challenging. Clinical symptoms are nonspecific and are easily overlooked. Furthermore, the sensitivity and specificity of diagnostic tests are debated, some tests are poorly tolerated, and for all there is a paucity of reference data from healthy infants and children.

Tests of adrenal reserve can be classified as those that test the integrity of the hypothalamic pituitary adrenal axis, including the insulin tolerance test, glucagon stimulation test, metyrapone test, cortico-releasing hormone test, and those that stimulate the adrenal gland directly, the Synacthen tests, which rely on involution of the adrenal gland in the absence of stimulation by adrenocorticotropic hormone.

Synacthen tests are used widely in pediatric practice, as they are safer and better tolerated than other tests that have been associated with fatalities [1]. Many different Synacthen test protocols are in use, but in general 2 broad dosing regimens are used, a "standard dose" (250 μg Synacthen, SDSST), and a "low-dose" (300 ng/m² body surface area to 1 μg, LDSST). The SDSST uses 500 times the dose of corticotrophin required for a stimulated cortisol response [2-4].The LDSST is as effective at producing a stimulated cortisol response as the SDSST in healthy individuals, and correlates well with results of the insulin tolerance test in adults [5]. Meta-analyses in pediatric and adult cohorts report the LDSST to be more sensitive but less specific for the diagnosis of AI than the SDSST [6-9]. However, inconsistency of dosing following the dilution of Synacthen to give the small doses required for children and young people (CYP) has been reported to be a critical weakness of this test [10] and evidence of the adherence of Synacthen to plastics has also raised concerns regarding the reliability of this test.

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The LDSST protocol used in our center since 2008 uses a dose of Synacthen calculated according to body surface area (500 ng/m²body surface area) [11]. Meta-analyses consistently report that the LDSST is more sensitive but less specific than the SDSST [6-9]. In our practice we elected to use the more sensitive test. Established LDSST protocols include sampling 0, 15, 20, 25, 30, and 35 minutes after Synacthen administration. Such frequent sampling is technically challenging in the smallest children. For this reason, the LDSST was simplified to reduce the intensity of sampling. Raw data from a large study of CYP with asthma, tested using a Synacthen dose of 500 mg/m²body surface area with sampling at 0, 15, 20, 25, 30, and 35 minutes, were reviewed [12, 13]. Sampling times that identified all CYP with a normal result were adopted for this simplified test. These data were used to design a protocol that allowed accuracy of sampling while improving patient acceptability.

We have reviewed the results of our cohort of CYP tested using the simplified LDSST regularly. We are aware of the risk of overdiagnosis and treatment of AI, given the concerns regarding the specificity of the LDSST. For this reason, we were interested in examining the number and characteristics of CYP diagnosed with AI requiring treatment with daily hydrocortisone. We investigated the relationship between age and sex, and baseline and stimulated cortisol concentrations as it has been suggested that age- and sex-specific reference ranges should be developed. It has also been reported that an early morning measurement of cortisol can be used to screen CYP for AI [8, 14, 15], and we therefore investigated the relationship between basal and stimulated cortisol.

To gain further understanding of the test, we grouped CYP according to clinical features that may indicate a higher or lower likelihood of a diagnosis of AI. For example, we anticipated that CYP with isolated fatigue in the absence of known risk factors for AI would be least likely to have an abnormal result, whereas those treated with an extended course of pharmacological doses of glucocorticoids were more likely to have AI.

The aims of this study were (1) to understand how the LDSST was used in routine clinical practice; (2) how often, according to departmental protocol [11], CYP were prescribed hydrocortisone either daily or during periods of stress only; and (3) to examine for relationships between cortisol measurements and indication for testing, age, and sex.

Materials and Methods

The protocol was reviewed by and registered with the hospital audit committee (reference No. 6134).

Population

The results of LDSSTs performed in CYP attending the day care unit or during an inpatient admission in our tertiary children's hospital between 2008 to 2014 (72 months) and 2016 to 2020 (42 months) were reviewed. Each patient was included only once in the data set. If a child had more than one LDSST, only the first test was included.

The following data were collected retrospectively: age, sex, indication for the LDSST, and cortisol concentrations at each time point.

CYP were classified into the following groups: respiratory disease requiring treatment with inhaled corticosteroids (ICS); completion of an extended course of pharmacological doses of glucocorticoids for other conditions (iatrogenic AI) including gastrointestinal, malignant, rheumatological, and renal disease; structural brain lesions involving the hypothalamus and/or pituitary diagnosed before the LDSST; poor cortisol response (peak cortisol < 450 nmol/L) to a growth hormone (GH) stimulation test performed for the assessment of short stature (insulin tolerance tests and glucagon stimulation tests); infants younger than 1 year with clinical features of AI; fatigue in the absence of known risk factors for hypothalamic, pituitary, or adrenal pathology; and CYP tested because they had one or more autoimmune endocrine pathologies known to be associated with AI, in whom clinical assessment by the lead consultant endocrinologist deemed testing to be appropriate, and "miscellaneous" reasons [11]. Infants were tested when a random cortisol was low during critical illness, prolonged jaundice, recurrent hypoglycemia, and following high-dose glucocorticoid use in intensive care.

Details of other pituitary hormone deficiencies were recorded for CYP with structural brain lesions, diagnosed with AI on the LDSST.

CYP with an abnormal cortisol response to a GH stimulation test were subclassified as those with growth hormone deficiency (GHD) and those with a normal GH response. CYP treated with ICS were tested if they were prescribed a dose of more than 800 μg beclomethasone equivalent/day, or CYP on more than 500 μg of fluticasone for more than 6 months, had received frequent "rescue doses" of oral prednisolone, or had symptoms of AI including poor growth, significant gain in body mass index, or excessive fatigue. The first time period included some CYP recruited to one of our previous studies of adrenal function in CYP with asthma [16], which also recruited CYP on lower doses of ICS and those without symptoms of AI.

Data were analyzed as a single group and in diagnostic groups that included more than 50 CYP.

Low-dose Short Synacthen Test Procedure

Details of the LDSST procedure are given within DataCat: the research Data Catalogue at the University of Liverpool [11]. In brief, the day of the investigation, an indwelling venous catheter was sited following the application of local anesthetic cream. A blood sample was collected (time 0). Body surface area was calculated from weight, and the dose of Synacthen was determined from a standard table [11]. A total of 125 μg (0.5 mL) Synacthen (Alliance) 0.25 mg/mL solution was added to 500 mL 0.9%Nacl (final concentration of 250 ng/mL) and agitated. A total of 500 ng/1.73m² body surface area was administered as a bolus injection directly into the cannula, and samples were collected 15, 25, and 35 minutes following Synacthen administration.

Assays

Serum cortisol was measured in the clinical laboratory at Alder Hey Children's Hospital by immunoassay using the Siemens Immulite 2000XPi immunoassay system (Siemens Diagnostics), an automated immunoassay analyzer using reagents supplied by the manufacturer. This assay has an intra-assay and interassay coefficient of variation of less than 5% and less than 7%, respectively. The same assay was used throughout this time period.

Interpretation of Cortisol Responses to the Low-dose Short Synacthen Test

CYP were classified into 1 of 3 groups on the basis of the baseline and peak cortisol measurement: (1) baseline cortisol greater than or equal to 100 nmol/L and peak cortisol greater than or equal to 450 nmol/L were considered to be at very low risk of AI and were classified as "normal"; (2) peak cortisol concentrations in the range of 350 to less than 450 nmol/L were considered to be "suboptimal"; and (3) those in whom peak cortisol was less than 350 nmol/L, who we considered to be a greatest risk of adrenal crisis, were described as having values that were "abnormal." These values were ascertained from a review of case reports and case series reporting adrenal crisis during ICS therapy in childhood [17-22]. Other than one patient, all other CYP tested in the nonacute setting had a peak cortisol of less than 350 nmol/L and generally below 200 nmol/L. For this reason, a cutoff value of peak cortisol of less than 350 nmol/L was identified to determine those at greatest risk of adrenal crisis. Previous literature [23] reports that a peak cortisol of 500 nmol/L in short, healthy children has a false-positive rate of 21% for the diagnosis of adrenal insufficiency, while a specificity of 94% has been reported, using a peak of 415 nmol/L. These data informed our definition of a "normal" peak cortisol being greater than or equal to 450 nmol/L.

CYP with a suboptimal response were treated with hydrocortisone (20 mg/m²/day) on "sick days" only, defined as days that the child was unwell enough to be kept off nursery or school, or injuries severe enough to need hospital treatment and surgical procedures. Those with an abnormal result were treated with maintenance hydrocortisone (8-10 mg/m²/day) and sick-day doses. If the diagnosis remained unknown after failing the LDSST, further investigations were undertaken to assess the cause.

Statistics

Data were recorded in Microsoft Excel and analyzed using R version 4.0.2 [24, 25]. A value of 50 was imputed for CYP whose baseline cortisol was below the limit of detection (50 nmol/L). The relationship between baseline cortisol and peak cortisol showed different gradients for lower vs higher baseline cortisol concentrations. For this reason, quantile regression techniques [26] were used to estimate the fifth and 95th percentile levels of peak cortisol for different values of baseline cortisol. Relationships were assessed using linear regression and adjusted for explanatory variables as required.

Results

Demographics

Data from 494 CYP were analyzed. The mean age was 9.5 ± 5.2 years and 295 (61%) were male. All samples across both time periods were amalgamated as there were no changes in the study protocol or in the assay during this time period. Patient characteristics are summarized in Table 1.

Cortisol Responses to the Low-dose Short Synacthen Test

For the group analyzed as a whole, mean (± SD) baseline cortisol was 221 ± 120 nmol/L, peak cortisol 510 ± 166nmol/L, and cortisol increment was 210 ± 116 nmol/L. A total of 336 (70%) CYP were classified as having a normal response, 78 (16%) a suboptimal response, and 67 (14%) an abnormal response. Table 2 summarizes cortisol responses to the LDSST as a whole and by subgroup.

Age and Sex by Diagnostic Group

Fig. 1 shows a box plot of the age distributions of each of the diagnostic groups (excluding infants). Age differed significantly between groups ($P < .001$, based on Kruskal-Wallis test), with CYP with autoimmune conditions and those with isolated fatigue being older.

There is also evidence of heterogeneity with respect to sex across diagnosis groups (Pearson chi-square test, $P = .02$; see Table 1). There are higher proportions of male CYP with GHD and in the miscellaneous category and higher proportions of female CYP in the structural brain abnormality and autoimmune disease groups.

Relationship Between Baseline and Peak Cortisol

The relationship between baseline cortisol and peak cortisol is illustrated in Fig. 2 showing quantile regression estimates of the fifth percentile (q_5) and 95th percentile (q_{95}) of peak cortisol as a function of baseline cortisol.

The fifth and 95th percentile levels of peak cortisol for different values of baseline cortisol are detailed in Table 3. These data can be used to predict peak cortisol based on baseline cortisol. For example, if baseline

Table 1. Characteristics of children and young people undergoing the low-dose Synacthen test by diagnostic category

Indication for test (No.)	Percentage of all tests in period	Age, y	Male, n (%)
Whole group (481)	100	9.5 ± 5.2	295 (61)
Inhaled steroids (ICS) (106)	22	10.5 ± 3.6	66 (62)
Pharmacological doses of steroids, not ICS (40)	8	9.7 ± 5.8	20(50)
Structural brain abnormality (136)	28	9.4 ± 1.0	77 (57)
Poor cortisol response to GH stimulation test (29)	6	8.2 ± 4.5	19 (66)
GHD (27)	6	10.6 ± 3.5	21 (78)
Infants (35)	7	0.3 ± 0.3	24 (69)
Fatigue (37)	8	11.7 ± 4.5	19 (51)
Associated autoimmune conditions (13)	3	15.1 ± 1.9	6 (46)
Miscellaneous (58)	12	9.5 ± 5.4	43 (74)

Data are shown as mean (± SD).
Abbreviations: GH, growth hormone; GHD, growth hormone deficiency; ICS, inhaled corticosteroids.

cortisol was 200 nmol/L, the fifth percentile would be 255.8 [(200 × 0.909) + 74] and the 95th percentile would be 762.8 nmol/L [(200 × 0.641) + 634]. If baseline is 200 nmol/L, there is a 90% probability that the peak cortisol concentration will be between 255.8 and 762.8 nmol/L. The analysis can also be used to determine the estimated level of baseline cortisol for which the fifth percentile of peak cortisol is equal to a given value. For instance, it is estimated at a baseline cortisol level of 418 nmol/L (95% CI, 384-435), there is a 5% chance of a peak cortisol level below 450 nmol/L. The inclusion of quadratic terms in the quantile regression was investigated but did not result in a statistically significant improvement of fit.

All CYP with a baseline cortisol greater than or equal to 419 nmol/L had a peak cortisol value of greater than or equal to 450 nmol/L. In contrast, one patient whose baseline cortisol value was below the limit of detection (< 50 nmol/L) had a peak cortisol value of 673 nmol/L. As such, there is no baseline cortisol value below which all CYP in the data set had a peak below 350 nmol/L, the value at which the CYP would be classed as failing the LDSST.

Baseline Cortisol

Relationship of baseline cortisol levels with age and sex

Age was related to baseline cortisol. Baseline cortisol increased by 2.7% (95% CI, 1.8%-3.7%) per 1-year increase in age. This relationship persisted after diagnostic group and sex were included as explanatory variables within the linear regression, although the magnitude of the effect decreased to 1.9% per year ($P < .001$; 95% CI, 0.8%-3.0%).

Baseline cortisol measurements were 11.5% higher in girls than in boys ($P = .03$; 95% CI, 1.1%-23.1%).

Differences between diagnostic groups

Differences in mean baseline cortisol values were observed between diagnostic groups, even after adjusting for differences in age and sex (F-test $P = .006$). GHD and CYP with an autoimmune condition associated with AI had the highest baseline cortisol values, with infants and those with structural brain abnormalities having the lowest values.

Peak Cortisol

Relationship of peak cortisol levels with age and sex

There was no effect of age on peak cortisol (either with or without controlling for diagnostic group). However, there was a statistically significant difference with sex, with girls having peak cortisol levels 60 nmol/L (95% CI, 31.4-88.6; $P < .001$) higher than boys after adjusting for diagnostic group and age. This effect also persisted after including baseline cortisol as a predictor for peak cortisol (effect of sex 45.7 nmol/L [95% CI, 20.15-71.22; $P < .001$] if age, diagnostic category, and baseline cortisol level was included).

Differences between diagnostic groups

Peak cortisol values between diagnostic groups differed, even after adjusting for differences in age, sex, and including baseline cortisol as a predictor (F-test $P < .001$). The groups with the lowest peaks are those treated with pharmacological doses of glucocorticoids, with structural brain abnormalities and infants, while the highest peaks occurred in CYP with isolated fatigue, a diagnosis of GHD in the absence of symptoms of AI, and those with autoimmune disease. All CYP who failed the LDSST who had a structural brain lesion had at least one additional pituitary hormone deficiency [11].

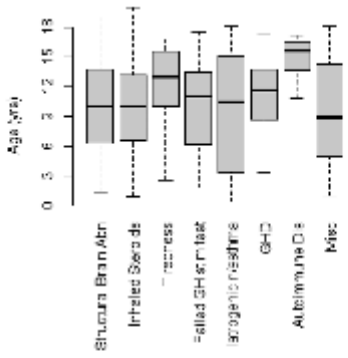

Figure 1. Box plot of age distribution by diagnostic group.

Table 2. Baseline and stimulated cortisol responses to the low-dose Synacthen test

Indication for test (No.)	Baseline cortisol, nmol/L	Peak cortisol, nmol/L	Cortisol increment, nmol/L	Normal, %	Suboptimal, %	Abnormal, %
All CYP (481)	221 ± 120	510 ± 166	210 ± 116	336 (70)	78 (16)	67 (14)
Inhaled steroids (106)	192 ± 94	431 ± 123	241 ± 120	59 (56)	26 (24)	21 (20)
Iatrogenic not ICS (40)	197 ± 91	410 ± 150	216 ± 145	20 (50)	8 (20)	12 (30)
Structural brain abnormality (136)	241 ± 125	553 ± 125	229 ± 121	108 (79)	15 (11)	13 (10)
Poor cortisol response to GH stimulation test (29)	236 ± 86	649 ± 88	391 ± 104	26 (90)	3 (10)	0(0)
GHD (27)	241 ± 85	639 ± 137	382 ± 154	22 (81)	4 (15)	1 (4)
Infants (35)	183 ± 188	465 ± 249	282 ± 224	13 (37)	9 (26)	13 (37)
Fatigue (37)	236 ± 117	583 ± 85	347 ± 134	36 (97)	1 (3)	0 (0)
Autoimmune (13)	305 ± 185	609 ± 185	307 ± 137	10 (77)	3 (23)	0 (0)
Miscellaneous (58)	214 ± 109	525 ± 170	317 ± 162	42 (72)	9 (16)	7 (12)

Abbreviations: CYP, children and young people; GH, growth hormone; GHD, growth hormone deficiency; ICS, inhaled corticosteroids.

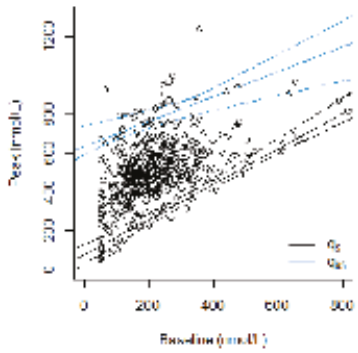

Figure 2. Quantile regression estimates of the fifth percentile (q5) and 95% percentile (q95) of peak cortisol as a function of baseline cortisol. Dashed lines indicate pointwise 95% CIs.

Table 3. Estimated model parameters for quantile regression models for percentiles of peak cortisol as a function of baseline cortisol

Model for 5th percentile	Estimate	95% CI
Intercept (α_1)	74.01	47.18-98.41
Slope (β_1)	0.909	0.866-0.959
Model for 95th percentile	**Estimate**	**95% CI**
Intercept (α_2)	634.6	597.7-725.3
Slope (β_2)	0.641	0.460-0.895

Increment in Cortisol Concentration

Relationship of incremental increase in cortisol concentration levels with age and sex

Age was associated with a decrease in the increment in cortisol concentration between baseline and peak (5.8 nmol/L reduction per year of age [95% CI, 2.9-8.6; $P < .001$]) as anticipated, given the relationships found in baseline cortisol and peak cortisol. The increment in cortisol was 28.9 nmol/L (95% CI, 3.2-54.7; $P = .03$) higher in girls than boys after adjusting for age and diagnostic category.

Differences between diagnostic groups

Mean increment in cortisol also differed between diagnostic groups (F-test $P < .001$), with those treated with pharmacological doses of glucocorticoids and structural brain abnormalities having the smallest increments and those with isolated fatigue and those with autoimmune disease having the largest increments.

Discussion

We believe this is the largest data set of cortisol profiles in CYP undergoing the LDSST reported to date. Analysis of such a large cohort has enabled relationships between baseline and peak cortisol concentrations and indication for testing, age, and sex to be explored. We see trends reported in previous work, including higher concentrations of cortisol in girls and older children [27, 28].

In this cohort of CYP, baseline cortisol concentrations could not be used to identify CYP who pass or fail the LDSST in a clinically helpful manner. It is likely that the baseline sample, collected as close to 9 am as possible, did not capture the peak of the cortisol awakening response in most CYP, and we did not document the time of waking the day of the test. Collecting a blood or saliva sample at home, half an hour after waking, may identify the true peak cortisol of the awakening response and be more informative.

Baseline cortisol was related to age and sex, with older children having modestly higher baseline cortisol concentrations than younger children, with an incremental increase with each 1-year increase in age. These observations are consistent with previous studies [28]. Peak cortisol was not affected by age, and correspondingly, incremental change in cortisol levels from baseline to peak decreased as age increased. Although these differences reach statistical significance and may be of interest in understanding the physiology of the maturing hypothalamic-pituitary-adrenal (HPA) axis, we suggest they are too small to justify the complexity of generating and using age-related reference ranges. These data are consistent with a previous study in a large cohort of CYP with asthma, treated with ICS [29].

Baseline and peak cortisol measurements and incremental increase in cortisol was higher in girls than boys. The difference in peak cortisol between girls and boys is similar to the sex difference we reported in an asthma cohort tested using the same LDSST protocol, 51.9 nmol/L higher in girls than boys (95% CI, –84.81 to –18.89; $P = .002$) [16]. This difference was not explained by age, and therefore pubertal status, or higher baseline cortisol concentrations.

We observed that those we anticipate being at greatest risk of AI (known structural brain abnormalities and following pharmacological doses of glucocorticoids) had the lowest cortisol concentrations at baseline and following Synacthen stimulation even after adjusting for differences in age and sex, and when including baseline cortisol as a predictor for peak cortisol. Our data do not allow us to comment on the sensitivity and specificity of the test in the absence of reference data from a healthy population, but it is reassuring that our observations make clinical sense.

It was anticipated that CYP treated with pharmacological doses of glucocorticoids for prolonged periods of time are likely to have had adrenal suppression. These CYP had been treated by specialists from other disciplines in our hospital, following their own weaning regimens before testing. Some patients underwent multiple tests during adrenal recovery. Only the first test was included in our study, and the period between the completion of glucocorticoid therapy and the LDSST is likely to differ considerably between CYP. High rates of adrenal suppression have been described in CYP following prolonged glucocorticoid therapy [30-32], and recovery of the HPA axis in CYP treated with ICS using this test protocol has also been described [29].

After CYP with iatrogenic AI, CYP with structural brain lesions were most likely to have AI. Each child who was diagnosed with AI via this test had evidence of another pituitary hormone deficiency, which is consistent with the sequence of pituitary hormone loss, in which adrenocorticotropin (ACTH) deficiency is generally preceded by GHD. In a previous cohort of CYP with brain tumors, 10.3% had evidence of ACTH deficiency [33]. This is similar to the number of CYP treated with daily hydrocortisone therapy on the basis of the LDSST result in our cohort, which also included CYP with congenital

defects of the pituitary, or following traumatic brain injury who may be at greater risk of ACTH deficiency [34].

CYP with an impaired cortisol response to a GH stimulation test were evaluated further with the LDSST. Most CYP in this category had a normal GH response and had undergone a glucagon stimulation test. If the child had undergone an insulin tolerance test, the LDSST was performed only if the child had no symptoms of cortisol deficiency. In this cohort of CYP, 20 (87%) CYP had a normal LDSST result, suggesting that the glucagon stimulation test may be less specific for the diagnosis of AI, and we have since revised the threshold at which the peak cortisol is considered to be abnormal on the glucagon stimulation test from greater than 550 nmol/L to greater than 350 nmol/L [35].

In a previous study, using basal serum cortisol of less 198.7 nmol/L with no significant increase during the insulin tolerance test, a much higher prevalence of ACTH deficiency was reported in CYP with GHD and a normal pituitary on magnetic resonance imaging [35]. This is likely to reflect differences in the patient cohort and diagnostic tests.

The difficulties of diagnosing AI in infants, in whom a number of perinatal factors are likely to influence activity of the HPA axis, is well documented. In this cohort we found baseline and peak cortisol concentrations were lower in infants than in all other diagnostic categories, other than those with structural brain abnormalities. In a previous study, 43% of infants demonstrated an abnormal response to a SDSST [36], very similar to the number of infants treated with daily hydrocortisone in our cohort of CYP. In this previous study, 69% of infants with an abnormal result had no identified pathology, and 90% of these CYP had a normal response to the SDSST at a later date [36], emphasizing the importance of repeat testing in infants.

Peak cortisol was highest in those CYP with fatigue, but no other clinical symptoms or signs of AI, no known pituitary or hypothalamic lesion, and no known risk factors for AI. The majority of CYP in this group had a normal response to LDSST. These data give some insight to the likely specificity of the LDSST and suggest testing for AI is unlikely to be informative in this group of CYP. We observed similar results in the group of CYP who were tested routinely, because they had one other autoimmune condition known to be associated with AI, but were not necessarily symptomatic. These data suggest that testing should only be performed in CYP with additional features of AI.

It is a weakness of this study that there are no reference data from healthy CYP. It is difficult to undertake invasive studies in healthy child volunteers, and our definitions of a "normal," "abnormal," and "suboptimal" response have been derived from historical data from cohorts of CYP with asthma [12, 29]. Therefore, it is not possible to state with confidence whether we have accurately underdiagnosed or overdiagnosed AI. However, the relatively small number of CYP treated with daily hydrocortisone on the basis of the LDSST, and the clustering of CYP with an abnormal result in diagnostic groups we consider to be at greatest risk of AI, suggest that overdiagnosis and treatment is unlikely to be very common.

All LDSSTs were performed by endocrine nurse specialists in a single medical day care unit, or on inpatient wards, according to a protocol [11] that is carefully designed to address concerns regarding the reliability of dosing and the risk of absorption to plastics. It is likely that results would be less reproducible in centers where the test is performed less commonly, or by less specialized staff.

The development of the salivary Synacthen test is an extremely positive development, as the use of noninvasive testing is likely to enable the development of reference ranges in the pediatric population.

Conclusion

Data from this large cohort of CYP show the results of the LDSST are consistent with previous studies, that the risk of overdiagnosis and treatment of AI in this clinical setting is probably low, and that clinical acumen is important in the selection of CYP that do and do not require testing. The number of CYP treated with daily hydrocortisone was modest and, in most diagnostic groups, not dissimilar from data reported previously.

We suggest the uncertainty rests in the group of CYP with a "suboptimal" test result, in whom we recommend hydrocortisone during periods of stress only. This conservative approach was adopted with the introduction of the LDSST protocol to avoid unnecessary daily treatment, but also to protect against adrenal crises in CYP with a peak cortisol response between the new threshold (350 nmol/L) and historical thresholds (450-500 nmol/L). We do not have strong evidence for this recommendation, and further work needs to be done to refine these thresholds.

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Disclosures

The authors have nothing to disclose.

Data Availability

Some data generated or analyzed during this study are included in this published article or in the data repositories listed in "References." Restrictions apply to the availability of some data generated or analyzed during this study to preserve patient confidentiality. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.

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